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The ecology of Listeria monocytogenes in ready-to-eat (RTE) meats

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The ecology of *Listeria monocytogenes* in ready-to-eat (RTE) meats

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For the Major Program

To Mom and Dad with all the love and support

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ABSTRACT

Survival and recovery of *Listeria monocytogenes* in the ready-to-eat (RTE) meat processing environment were studied, from environmental contamination to intervention. This pathogen survived in simulated dust using sand for more than 70 d. Longer daily survival rate and better recovery were obtained when inoculated sand samples were stored at lower temperatures with higher relative humidity levels. *L. mononocytogenes* survived and grew once in contact with RTE meats. No significant differences ($P > 0.05$) in attachment were observed for the different selected RTE meats. Desiccated cells of *L. monocytogenes* were not observed to go into viable but non-culturable (VBNC) state well indicating that current methods of using nonselective media, tryptic soy agar with 0.6% yeast extract, is adequate in recovering the majority of the viable cells. Addition of pyruvate was comparable but anaerobic incubation had less recovery. Five individual strains and a mixed cocktail of all five showed no significant differences ($P > 0.05$) in attachment characteristics. *L. monocytogenes* (86%) attached strongly to RTE meats within 5 min. No significant differences ($P > 0.05$) were observed in cell surface hydrophobicity and cell surface charge among strains. However, using cationized ferritin, culture age affected the cell surface charge. Processed meats have a relatively hydrophobic surface as observed from contact angle measurements. Once *L. monocytogenes* was inoculated onto the surfaces, the angles were altered. Low dose irradiation to provide safety while minimizing organoleptic changes is possible. A dose of 1.5 kGy was established for 3-log reduction while 2.5 kGy for 5-log reduction of *L. monocytogenes* contaminated RTE meats based on nonselective media. Preliminary results showed no growth in meats irradiated at 4.0 kGy. Survivors were

observed for irradiated meats at 2.0 kGy stored at 10°C after the second week. No growth was observed in samples irradiated at 2.0 kGy stored at 4°C until the fifth week. A sensory evaluation was conducted with meats irradiated at 1.5 kGy. Consumers could not detect any differences ($P > 0.05$) between irradiated and nonirradiated frankfurters but differences ($P < 0.05$) were detected between irradiated and nonirradiated sliced meats.

CHAPTER 1.

GENERAL INTRODUCTION

Listeria monocytogenes-contaminated processed meats have been incriminated as a source of a number of foodborne outbreaks recently. Since processing of ready-to-eat (RTE) meats such as sliced roast beef, turkey ham, bologna, or frankfurters, includes high temperature cooking, the product should be safe to consume. Moreover, these products contain antimicrobial ingredients, which include sodium chloride, nitrates, nitrites, phosphates, and constituents of smoke. In terms of processing, processed meats are cooked, smoked, and vacuum packaged, adding safety to products. Since *Listeria* does not survive cooking (160°F), contamination of *Listeria* usually occurs during post-processing and handling of these products (peeling, slicing, and packaging) as this bacterium is common in the environment. Possible sources of this environmental contamination can be the surrounding physical environment, which include dust, airborne moisture, or condensate, and the product itself that may be in direct contact with processing equipment or packaging films. The actual mechanisms of bacterial contamination are poorly understood. Further research is needed to elucidate how *L. monocytogenes* attaches to processed meats, survives, and proliferates, thus potentially causing illness. Once the mechanism of attachment and proliferation of *L. monocytogenes* in RTE meats is better understood, it is likely that effective measures can be taken to control contamination.

One measure to control contamination is use of irradiation. Irradiation at doses to provide safe foods while minimizing loss of organoleptic qualities need to be understood. Other concerns include the possibility of survival and outgrowth of *L. monocytogenes* using this low-dose irradiation on RTE meats. In order to understand consumers' responses, a

sensory evaluation is needed to determine if they could tell a difference between low-dose irradiated processed meats and those without this treatment.

Dissertation Organization

This dissertation has six chapters. Chapters 2 to 5 are four manuscripts. Chapter 1 covers general introduction, dissertation organization, and literature review. Chapter 2 describes the occurrence and recoverability of viable but nonculturable (VBNC) *L. monocytogenes* cells. Chapter 3 discusses the mechanism of *L. monocytogenes* attachment to RTE meats with emphasis on cell surface charge and cell surface hydrophobicity. Use of irradiation as an intervention for *L. monocytogenes* contamination on RTE meats is focused in Chapter 4. Determinations of D_{10} values, doses required for safety, and survival and outgrowth are discussed. The last manuscript, Chapter 5, discusses the sensory evaluation of RTE meats as a result of the irradiation doses found from the previous chapter. Chapter 6 contains general conclusions and recommendations for future research.

Literature Review

Foodborne listeriosis is rare but severe (*285*). *Listeria monocytogenes* is estimated to cause 2,518 illnesses, 2,322 hospitalizations, and 504 deaths in the United States, and these numbers are believed to be under-reported (*169, 301*). About 92.2% of cases require hospitalizations and the case-fatality rate is 20% (*189, 260*). The national health objective (Healthy People 2010) is to reduce the foodborne listeriosis from 0.5 to 0.25 cases per 100,000 and the Foodborne Diseases Active Surveillance Network (FoodNet) is targeting *L. monocytogenes* as a pathogen of public health concern (*44, 45, 87, 97, 301*). *L.*

monocytogenes has been recently associated with several outbreaks where RTE processed meats are incriminated as the source. The primary concern is that this pathogen is a post-processing contaminant and most often these RTE meats are consumed without any prior cooking. Ingestion of this pathogen causes illnesses and even death to people who are susceptible.

Listeria monocytogenes

Thirteen serovars exist within the species *L. monocytogenes* (*94, 306*). The most predominant in the United States is 4b while 90% of the infections worldwide are associated with 1/2a, 1/2b, and 4b (*94, 306*). This non-spore forming bacterium is resistant to freezing and drying (*144*). *L. monocytogenes* tolerates low temperatures (-0.4°C) and a wide range of pH between 3.6 and 9.6 (*34, 56, 84, 100, 110, 128, 144, 169, 176, 287*). This microorganism also tolerates relatively low water activity (a_w) >0.90 (0.936) and high salt (>10%) concentrations (*9, 56, 67, 84, 100, 110, 128, 144, 169, 176, 287*). Previous researches have shown this pathogen to survive -23°C for 20 days in mutton, -20°C for 2 years with 35 thawings, and -18°C for 70 days in butter (*100*). This psychrotolerant nature is associated with a decrease in fatty acid chain length (*136*). *L. monocytogenes* appears to survive in processed meats by accumulating osmoprotectants, glycine betaine and proline, to overcome growth inhibition by osmotic stress. Peptone, a nutritional supplement for protein synthesis and a source of amino acids and peptides, maintains cell turgor in osmoregulation (*9*).

Taxonomy and Phylogeny

Listeria monocytogenes is identified and named in 1924 as *Bacterium monocytogenes* (*232*). Other names include *Bacillus hepatis* and *Listerella hepatolytica* (*94*). Finally, the name *Listeria monocytogenes* is given in honor of Sir Joseph Lister and 'monocytosis' observed in infected rabbits (*166*). *L. monocytogenes* is one of the species in the genus *Listeria*. Other species include *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, and *L. grayi*. The phylogenetically closely related *Brocothrix* is differentiated by its inability to grow at 35°C and lack of motility (*120*). *Listeria* is morphologically similar to corynebacteria but genetically related to and grouped under *Lactobacillus* (*306*). Surface protein patterns show high degree of homogeneity between all strains of the same serovar but large differences between serovars, particularly between 1/2 and 4b (*273*).

Morphology, Culture Characteristics, and Metabolism

L. monocytogenes is a Gram-positive coccobacillus, occasionally occurring in short chains (*52*). It utilizes esculin, which antagonizes iron-chelating agents (*52*). Cell size varies from 0.4 to 0.5 μm by 0.5 to 2.0 μm with rounded ends. Four peritrichous flagella are present, resulting in a tumbling run and motility at 20 to 25°C, but only one polar flagellum is present when the bacterium is grown above 30°C. All *Listeria* spp. are negative for indole, oxidase, urease, and hydrogen sulfide (H_2S) production, but positive for catalase, methyl red and Voges-Proskauer. Beta-hemolysis is produced on sheep blood agar. Optimum temperature for growth is 37°C. This bacterium is facultative anaerobic but growth is improved when incubated with reduced oxygen and 5 to 10% carbon dioxide (CO_2) (*12, 73, 112, 120, 236, 287, 306*).

Injured and Stressed Cells

Healthy cells such as those cultured in the laboratory with rich media, attach to beef tissue in more numbers than starved cells (*70*). However, bacteria in natural environments are exposed to nutrient fluxes, leading to a starvation-survival state defined as a physiological condition resulting from insufficient nutrients supplying energy for growth and reproduction (*141*). Stationary-phase cells are more resistant to stresses caused by the production of intracellular free radicals than those in exponential growth phase (*242*). Cells of *L. monocytogenes* in the environment are stressed structurally or metabolically by sanitizers (amines, quaternary ammonium compounds, and peroxides), preservatives, heat, cold or freezing, drying, osmotic shifts, and acid (*16, 35, 53*). Exposure to stresses causes 'stress hardening', the development of 'stress-adapted' strains having increased resistance to normal levels of homologous or heterologous (cross-protection) inimical stresses (*101, 242, 267*). This resistance can be acquired through the production of stress proteins, mutation, or genetic transfer such as transformation, transduction, or conjugation (*28, 118*).

Temperature and nutrient depletion affect the physiology of *L. monocytogenes*. Heat inactivates enzymes (dehydrogenases) and destroys 16S rRNA causing lethal damage to the cells (*35*). Strain, age, growth condition, recovery media, and food characteristics influence heat resistance (*74*). Damages caused by chill storage (>4 weeks) include cytoplasm shrinkage and cell wall damage (*77*). Cold stress changes attachment capabilities, reduces pathogenicity, and reduces growth rate (*78*). Freezing and thawing *L. monocytogenes* causes cell wall and plasma membrane damages due to formation of intra- and extracellular ice crystals (*35, 77*). Survival from starvation occurs by obtaining energy endogenously from storage materials, RNA, and protein to maintain cellular processes (*141*). Starvation stress

includes alterations of surface properties, cell morphology, membrane phospholipids structure, metabolic activity, and protein composition. An increase in cell number, decrease in cell size, increase in cell surface hydrophobicity, increase in adhesiveness, and production of stress proteins may also be present with stress (*118, 226, 262, 300*). Cells of *Listeria* grown in low nutrient concentrations generally show more resistance towards stresses than the ones grown in high nutrient concentration (*57*). Protein synthesis, mRNA, and oxidative phosphorylation may be required for repair to occur (*35*).

In response to chemical or environmental stress, stimulon genes produce proteins for protection. Examples of stimulons include heat shock and oxidative stress (*262*). Injured cells can repair sublethal damage and regain growth and virulence potential (*35*). GroEL and chaperonins counter stresses by assuming protein folding, renaturation, and damaged proteins evacuation (*225*). Stressed *L. monocytogenes* initiates the starvation survival response (SSR) involving protein and cell wall biosynthesis (*118*). Uptake of carnitine, proline, and glycine betaine, osmolytes in meats and plants, act as osmoprotectants and resist acid and chill stress by maintaining cell turgor pressure (*79, 80, 114, 261*).

In RTE meats, salt is used for curing and together with pH, a strong synergistic effect occurs that produced cell filamentation and make cell surfaces hydrophilic (*16*). In growth experiments, increased osmotic pressure and acidic and alkaline conditions increase log phase and decrease growth rates of *L. monocytogenes* (*292*). Cells recovered from alkaline stress grow more rapidly than the ones recovered from acid and osmotic stresses (*48*). Osmotic stress-adapted strains are more thermotolerant and low pH adapted cells survive longer under acidic conditions (*24*). Upon exposure to acidic conditions, *L. monocytogenes* acquire an acid tolerance response (ATR), through an adaptation period, during exponential

growth (*62, 242*). Acid tolerance is dependent on strain, growth phase, and type of acid. Organic acids are more lethal than inorganic ones by lowering cytoplasmic pH. Mild acids infer acid adaptation while extreme acids, acid stress (*225*).

Resuscitation and Recovery. Injured and stressed cells can be resuscitated to grow on nonselective media by adding nutrients. These cells will replicate a number of times before entering a 'dormant-like' state. Cells in this 'dormant-like' state require more complex conditions to resume growth and division (*241*). These cells need to synthesize membrane lipids and phospholipids, protein, ATP, ribosomal RNA, and repair breaks in the DNA (*76*). Survival increased at suboptimal growth temperatures with less pressure on the repair mechanism (*167*). Anaerobic incubation is found to give better recovery of heat-injured *L. monocytogenes* compared to aerobic incubation (*234*). Damaged cells from thermal processes or acidification are also sensitive to selective agents (*231*). Therefore, the *Listeria* repair broth (LRB) containing divalent cations (Mn, Mg, and ferric ions), amino acids for protein synthesis, and pyruvate, is suitable for injured cells to recover and facilitate repair (*35, 53*).

Sublethally-stressed bacteria are sensitive to oxygen tension, thus better recovery is obtained by plating on minimal media. This 'bacterial cell suicide' explains 'viable but nonculturable' state where stressed cells show normal respiratory and metabolic functions but cannot be cultured on standard media. In order to assist recovery, the addition of oxygen scavengers, reducing agents such as pyruvate, glutathione, or Oxyrase®, may be required (*242*). However, higher concentrations will limit recovery by forming $O_2^{\bullet-}$ with the media (*271*). Many techniques are available to resuscitate injured bacteria in foods (*290*).

Viable but Nonculturable (VBNC) Cells. Some stressed cells remain viable but unable to grow on media, therefore, underestimating viable cell numbers in the surviving population (*61, 167*). For example, *L. monocytogenes* exposed to benzalkonium chloride (BAC), a sanitizer, is not detected with plate counts but remained viable based on the direct viable count (DVC) method (*234*). This VBNC state has been observed in human pathogens such as *Escherichia coli*, *Salmonella enteritidis*, *Vibrio* spp., *Legionella pneumophila*, *Staphylococcus aureus*, *Bacillus subtilis*, *Campylobacter jejuni*, and *Shigella* spp. (*18, 62, 186, 218, 219, 237, 241, 252*). Nonculturable response over time is affected by starvation, temperature [intermediate (25 to 37°C) faster than lower (4 to 5°C)], sodium concentration, growth phase, and use of disinfectants (*19*).

The maintenance of virulence for cells entering VBNC is questionable (*218*). Virulence loss, reduced number of ribosomes per cell, increased short-chain fatty acid of the membrane, and reduced amino acid transport occur with temperature changes. Once nutrients are depleted, cells become more resistant to heat, chemical oxidative, and osmotic stresses (*186*).

Some VBNC cells can be resuscitated with warmer temperatures to the culturable state regardless of physiological state and encapsulation. Cells producing stress proteins in the stationary phase take longer to enter VBNC state compared to the ones in logarithmic state (*219*). The primary concern is that pathogens may regain culturability and potentially cause disease. However, no colonization is shown with VBNC cells of *C. jejuni* in embryonic eggs and 1-day old chicks (*190*).

A direct microscopic method has been developed to detect VBNC cells (*122, 147*). This technique has been used to test water samples in marine microbiology and to study

stressed bacterial cells in the environment including *L. monocytogenes* (*93, 147*). *L. monocytogenes* loses culturability but remain viable after 10 weeks under nutrient deprivation (*19*). Samples are pre-incubated with a DNA synthesis inhibitor (DNA gyrases), preventing cell division (DNA replication) but allow other synthetic pathways to proceed, resulting in elongated cells (*147, 233*). Examples of these DNA gyrase inhibitors include nalidixic acid for Gram-negatives and novobiocin, ciprofloxacin, enrofloxacin, enoxacin, norfloxacin, and isopropyl cinodine for Gram-positives (*18, 19, 93, 147, 233, 252*). Samples are filtered through the preferred nuclepore filters as higher populations are recovered in comparison to cellulose filters. These samples are stained with acridine orange and cell elongation increase from 0.8 to 1.5 μm to a length of 2.4 to 4.8 μm for *L. monocytogenes* (*27, 93, 122, 147*). Viable cells with abundance of cellular RNA bind acridine orange stain fluorescing reddish orange under fluorescent microscope. Dead cells with their lower concentration of RNA will fluoresce greenish white (*147*). Higher populations (half a log) are observed from this DVC method compared to population recovered on nonselective media and significantly more so than selective media (*93, 252*).

In addition to acridine orange, a commercially available kit (*Bac*light) stains live bacteria with intact membranes green and dead cells with disrupted membranes orange (*76*). Other methods of detecting VBNC cells include flow cytometry with fluorescent probes, fluorescent in situ hydridization (FISH) with 16S rRNA as the target, radiolabelling, and CTC-DAPI double staining to detect active electron transport chain (*19, 233, 283, 294*).

Sources, Transmission, and Isolation

L. monocytogenes has been found in animals (intestine), birds, marine life, insects (ticks), protozoans, soil, dust, feces, healthy or decaying plants, fertilizer, sewage, stream water, and silage. Humans (1 to 5%) are asymptomatic carriers (*12, 53, 57, 94, 100, 172, 269, 287, 306*).

Food associated with *L. monocytogenes* include frankfurters, deli meat, raw or improperly pasteurized milk, dairy products, raw vegetables, coleslaw, all types of raw, processed, fermented, and cooked meats, meat spreads (paté), jellied pork tongue, and raw and smoked seafood (salmon and sushi), vegetable salad, and pasteurized vegetarian meals (*7, 43, 57, 84, 97, 100, 110, 146, 178, 197, 212, 235, 254, 257, 287, 288, 306*). Listeriae has been detected in vacuum-packed processed meats at <10 up to >1000 cells/g and raw cured meat products (13.71%) are more contaminated than cooked ones (4.90%) in Belgium (*230, 288*). Food-to-human transmission occurs from infected animal wastes or unsanitary food handling (*212*). Exposure is dependent on the food amount ingested, frequency of consumption, populations of bacterium present, refrigeration storage temperature, moisture condition, and storage time prior to consumption (*67, 285*).

Conventional and Rapid Methods for Isolation

In microbiological analysis, excising sampling is less affective than swabbing (*296*). Blending and stomaching to homogenize samples are acceptable and as diluents, phosphate buffers yield lower counts than buffered peptone water or 2% trisodium citrate buffer (*66*).

Pre-enrichment of samples, especially environmental ones due to the low initial populations, is recommended. With low levels of contamination, 2 d incubation period in

enrichment broth shows better recovery than 1 d (*277*). Nonselective broths, namely brain heart infusion (BHI) and tryptic soy broth with yeast extract (TBSYE), stimulate listerial growth (*31, 270*). Available selective enrichment broths include Fraser broth, FDA enrichment broth (EB), *Listeria* selective broth (LSB), *Listeria* enrichment broth (LEB), University of Vermont LEB (UVM), and *Listeria* repair broth (LRB) (*53, 78, 212, 258, 277*). Samples are inoculated into the selective enrichment broth at 30°C for 48 h and streaked onto nonselective or differential selective agar (*120, 121*).

Selective agents for *L. monocytogenes* are phenylethanol, glycine, nalidixic acid, acriflavine, moxalactam, ceftazimide, lithium chloride, esculin, and ferric ion salt. A Dutch differential selective medium, PALCAM, consists of a base with selective supplements (*100, 120, 146*). Selective agars are modified McBride agar (MMA), acriflavin-ceftazidime (AC) agar, Oxford agar (OXA), and lithium chloride-phenylethanol-moxalactam (LPM) agar. Oxford agar shows the best recovery (*277*). Metabolites from esculin hydrolysis interact with ferric ions producing blackening of the medium (*53*). Metabolic end products of *L. monocytogenes* grown in a glucose-defined medium incubated aerobically are lactate, acetate, and aectylmethycarbinol (acetoin). During anaerobic incubation, lactate, acetate, formate, ethanol, and carbon dioxide are produced. Specific growth rates are similar but with the maximum cell density of 1 $\log_{10}$ higher in aerobic compared to anaerobic incubation. However, anaerobic incubation shows better detection of heat-treated *L. monocytogenes* survivors (*238, 266*).

Available rapid kits include enzyme linked immunosorbent assay (ELISA), DNA-probe kits, and chemiluminescent-labeled DNA probe kit (*120*). Molecular-based methods to diagnose *Listeria* are ribotyping, multilocus enzyme electrophoresis, pulse-field gel

electrophoresis (PFGE), polymerase chain reaction (PCR), DNA probe hybridization, analysis of DNA restriction endonuclease patterns, random amplified polymorphic DNA typing, and radiolabelling (*8, 18, 120*). The ELISA provides qualitative detection whereas ribotyping and PFGE differentiates individual strains of *L. monocytogenes*.

Listeriosis

The first reported human listeriosis is in 1929 (*100*). Listeriosis occurs as the bacteria migrate from intestinal lumen to the central nervous system (CNS) or the fetoplacental unit (*154*). Animal listeriosis manifests as septicemia, meningoencephalitis, encephalitis, and placentitis (spontaneous abortion) while asymptomatic healthy animals shed the organism in the feces (*299*). The most susceptible populations include pregnant women or fetus; immunocompromised persons (ex: corticosteroids impairing cell-mediated immunity, cancer and anticancer drugs, AIDS patients with $CD4^+$ T-lymphocyte counts $<40/mm^3$, diabetic, cirrhotic, asthmatic, renal transplant recipients); individuals with predisposing factors (antibiotics or antacids); young (<5 years); and elderly (>65 years) (*269, 287, 291, 306*). Animal-to-human and nosocomial transmissions have been documented (*87, 100, 116*). Human-to-human transmission occurs transplacentally through the umbilical cord (septicemia) or birth canal with infected secretions (*116, 306*).

L. monocytogenes is an opportunistic pathogen, for which the minimum infectious dose (MID) is unknown and depends on strain and victim susceptibility, perhaps $<10^3$ cells (*94, 228, 262, 287*). Some processed meats have contained populations of 10^3 to 10^4 cells/g or greater (*112, 177*). Adults acquire listeriosis when the food consumed is contaminated with 10^5 to 10^9 cells/g (*259, 287*). Clinical listeriosis is defined when the bacterium is

isolated from blood, cerebrospinal fluid, amniotic fluid, genital tract secretions, placenta or fetus (*287, 306*). Human manifestations include septicemia, bacteremia, meningitis, encephalitis, meningoencephalitis, endocarditis, granulomatosis infantiseptica, non-meningitic CNS infection, or intrauterine or cervical infections in pregnant women resulting in spontaneous abortion in the second or third trimester (*269, 287*).

Influenza-like symptoms (persistent fever) precede these disorders. Gastroenteritis with symptoms such as nausea, vomiting, and diarrhea, occurs with ingestion of up to 3 x 10^{11} bacteria/person (*154, 287*). Focal infections occur in the bones, joints, eyes, endocardium, spinal cord, peritoneum, and gall bladder (*269*). Time of onset of serious symptoms varies from few days to 8 weeks while gastrointestinal symptoms occur after >12 h (*42, 87, 287*).

Virulence and Pathogenicity

The major point of *L. monocytogenes* entry is the gastrointestinal tract (*110*). Primary targets inside a host are cells of mononuclear phagocyte system, enterocytes, gastrointestinal epithelium (lymphoid follicles), fibroblasts, the Peyer's patches, hepatocytes, and vascular endothelial cells. Normal microbial flora modulates gut colonization. As an intracellular pathogen, it can overcome host polymorphonuclear leukocytes, monocytes, or macrophages, becoming bloodborne (septicemic) with transplacental migration to the fetus or traveling to the brain (*94, 134, 269, 287, 306*).

Virulence factors are not well understood as *L. monocytogenes* lacks a capsule, fimbriae, or adhesins. The virulence may be explained by its survivability within macrophages and resistance to lysozyme (*102*). The cell wall contains a substance similar to

those of lipopolysaccharide (LPS) endotoxins in Gram-negative bacteria (*306*). Monocytosis-producing activity (MPA) of the plasma membrane reduces generation times of monocyte precursors (*102, 306*). Internalin (InlA), a virulence factor, mediates crossing from intestine into epithelial cells while Inl B mediates entry into hepatocytes, epithelial or fibroblast cells (*29, 94, 154, 269*).

The intracellular environment of macrophages is very stressful. The low pH activates hemolysin, a virulence factor, with an optimal pH at 5.5 and becomes inactivated at pH 7. This hemolysin, exotoxin listeriolysin (LLO), and phosphatidylinositol-specific phospholipase C (PI-PLC) form pores and disrupt phagocytic vacuole and lysosome membranes. The pathogen escapes phagosomes and enters the cytoplasm where they grow with a doubling time of about 30 min (*56, 82, 89, 94, 100, 102, 107, 251, 269, 306*). Polarization occurs in 2 h where the bacterium moves to the periphery pushing against the outer membrane of the host cell to form elongated protrusions (filopods), which are ingested by neighboring cells (*269*). The ActA protein affects actin polymerization by forming actin filaments or tails for propulsion and mediates cell-to-cell spread without contacting the extracellular environment. This intracellular and intercellular spread explains the escape from complement- and neutrophil-mediated immune defenses (*29, 56, 89, 94, 256, 269*).

Immunological Response and Interventions

Host defenses, which can overcome this intracellular infection, are phagocytic macrophages, activation of the alternative complement system and opsonization (*134, 306*). The innate immune system, blood monocytes and macrophages, neutrophils, natural killer (NK) cells, and T cells control populations during early infection. Tumor necrosis factor

(TNF) produced by macrophages and interferon (IFN)-γ produced by T_H cells eliminate *L. monocytogenes* (*59, 89, 102, 214*). Neutrophils destroy cells infected with listeriae and when listeriae are released from lysed cells (*56*).

The primary host defense is cell-mediated immunity (*269*). *Listeria*-specific CD4 T cells are capable of mediating delayed type hypersensitivity (DTH) reaction (*56, 137*). Cytotoxic CD8 (T_C) cells are essential in protective immunity lysing infected macrophages (*56, 214, 256*). Other activated macrophages restrict escape from phagolysosome, prevent intracellular multiplication, and increase macrophage inactivation of ingested listeriae. Antibodies targeting *L. monocytogenes* antigens are not protective but serve a minor role. With the complement system, killing by neutrophils and macrophages is enhanced (*56*).

Antibiotics such as penicillin G (with gentamicin), ampicillin, erythromycin, tetracyclines, and trimethoprim-sulfamethoxazole are effective and ampicillin and penicillin are the preferred antibiotics for treatment of illness. Aminoglycosides are ineffective, and trimethoprim-sulfamethoxazole is the most effective in killing *Listeria* (*56, 269, 306*). Natural *L. monocytogenes* resistance is found to fosfomycin (*282*). An antifungal treatment for immuno-compromised persons, amphotericin B, impairs target cell lysis and suppresses CD8 T cell function (*104*). Competitive exclusion, feeding yogurt with live cultures, out-compete pathogens for intestinal lining in animals (*28*). However, once a biofilm is established, *L. monocytogenes* maintains as 1% of the total population (*135*). As prevention, recombinant *L. monocytogenes* vaccines have been developed to protect against tumors or cancers, human immunodeficiency virus (HIV), lymphocytic choriomeningitis virus (LCMV), or influenza type A (*179, 217, 256*). Passive administration of antibody to LLO

imparts resistance to *Listeria* infection. Gene gun administration of plasmid DNA into the dermis induces immunity against *L. monocytogenes* (*82, 88, 221*).

Processed Ready-to-Eat (RTE) Meats

Processing meats preserve perishable meats and extend shelf-life. Additional advantages include flavor addition, convenience, preparation ease, portion control, and savings on labor and energy for the consumers. Forms of processed meats available include whole, subdivided, and restructured products (*140*). Salt changes the microflora from Gram-negative (pre-processing) to Gram-positive (post-cooking) for processed meats (*249*). Inoculated *Listeria monocytogenes* can survive storage at 4°C for a week on cured ham, dry cured bresaola, mortadella, and cooked ham (*231*). Retail wieners support growth of *L. monocytogenes* as the surface is composed of small cavities in a dense layer of coagulated protein and pH ≥6.0 (*187, 298*). Source of *L. monocytogenes* in cured dried meat products may be from fresh meat, where the incidence ranges from 0 to 92% (*211, 296*). *L. monocytogenes* survives fermentation and is not inactivated by traditional temperature procedures such as cooking (*280*). In handling cold cut meats, human contamination of *L. monocytogenes* often occurs with concerns over personal, especially hands, and general hygiene (*211*).

Formulations with Antimicrobial Properties

In addition to changes of the natural microflora, salt also preserves meat, extracts proteins for binding, and for flavoring. Salts and sugars are osmotically active substances to lower the water activity (a_w) of foods (*114, 140, 173*). Curing agents increase heat resistance

of *L. monocytogenes* (*181*). With ascorbate and sodium nitrite at permitted levels, *L. monocytogenes* is inhibited in refrigerated products containing at least 3% NaCl and pH <5.5 (*100, 110, 168, 266*). At low temperature storage, high residual nitrite or low a_w reduces listerial growth (*112*). Weak organic acids such as acetic, lactic, benzoic, sorbic, propionic, and phenolic compounds are common preservative agents (*33, 266, 298*). Effectiveness is acetic > lactic > citric > malic > hydrochloric at equal pH, however, the order changes at different temperatures (*266*). Ethylenediamine tetraacetic acid (EDTA), a chelator, helps weak acids against Gram-negatives to make the outer membrane more permeable (*33*). Organic acids are antimicrobic from the undissociated molecule, and not from the hydrogen ion, which interferes with microbial cell membrane permeability, substrate transport, and oxidative phosphorylation. Sodium benzoate and sorbic acid inhibit *L. monocytogenes* at lower temperature and pH (*216*). Sodium lactate (3 to 4%) and sodium diacetate, control listerial growth in processed meats (*60, 119, 131, 182, 201, 266*). Inhibitory activities of sodium lactate and sodium propionate increase with fat content and are listericidal at 4°C (*125*). Sodium lactate with lowered pH suppresses growth of *L. monocytogenes* in chilled cooked meat (*229*). A combination of sodium lactate and monoglycerides shows listericidal effects (*284, 295*). Dipping in preservatives for 1 min decreases slime formation and increases shelf-life (*130*). Sodium acid pyrophosphate (SAPP) show antimicrobial activities but the mechanism is unknown (*173*). A proprietary phosphate blend, Bekaplus MSP, does not inhibit this pathogen (*90*). Other ingredients in processed meat formulations, milk, casein, cane sugar, cocoa powder, and carrageenan, may also be inhibitory to *L. monocytogenes* (*266*).

Background flora or simultaneous growth of other microorganisms affects growth and survival of the pathogen due to nutrient competition and antimicrobial metabolites such as bacteriocins (*65, 229*). Starter cultures for dry sausage have been developed with bacteriocin-producing capabilities to control growth of *L. monocytogenes*. Starter cultures, lactic acid bacteria (LAB), are incorporated to control this pathogen in nonfermented cooked meat products without affecting organoleptic properties negatively. When *Pediococcus acidilacti* (10^7 CFU/g) is added to frankfurters, pediocin (a bacteriocin), is produced which is effective in controlling the growth of *L. monocytogenes* (*83, 192, 280*).

Post-heat processing organic acid dips for processed meats reduce *L. monocytogenes* (*220*). Acetic acid and citric acid, each at 2.5%, restrict growth of *L. monocytogenes* on vacuum-packed frankfurters stored at 5°C for 90 d (*83*). Acetic and tartaric acids are more inhibitory than lactic and citric acids (*100*). However, organic or inorganic acids to lower pH have no significant impact on *Listeria* adhesion (*75*). Innate resistance against antimicrobials includes cellular barrier and efflux, lack of biochemical target for attachment, and inactivation by microbial enzymes (*60*).

Processing of RTE Meats

L. monocytogenes does not survive normal cooking conditions. *L. monocytogenes* contaminates ham during tumbling but cooking to a proper internal temperature (70°C) and careful handling will render the final end product safe (*249*). Cooking is most effective in destroying spoilage and pathogenic bacteria and parasites. A temperature of 60°C for 3 to 9 min kills 90% of *Listeria* cells (*110*). When taking 70 min to reach an internal temperature of 160°F during the processing of frankfurters, this process kills *L. monocytogenes* in raw

meats (*305*). Smoking inhibits microbial growth and *Listeria* with its dehydrating effect (*140, 168, 266*).

Packaging and storage temperature may control contamination of *L. monocytogenes*. Generally, modified atmosphere packaging (MAP) and controlled atmosphere packaging (CAP) methods extend the shelf-life of cooked meat products. However, *L. monocytogenes* can survive in this anaerobic condition, multiplying and growing, potentially posing a threat to consumers (*57, 65*). *L. monocytogenes* growth on luncheon meat, ham, and chicken breast is similar under vacuum and modified atmosphere (30% CO_2/70% N_2) packaging (*20*). Increased growth occurs with an increase in either pH or temperature on fatty tissue (*111*).

Temperature abuse, for example, storing these perishable RTE meats at room temperature due to temperature control failure, can occur during distribution and in retail markets (*148*). Therefore, refrigeration is critical. It extends shelf-life of meats but increases risk in growth of surviving psychrotrophic pathogens and cross-contamination of cooked products (*111*). Chilled storage causes sublethal damage and reduces pathogenicity of *L. monocytogenes* (*81*). With processed meats, storage at temperature <5°C maintains product quality. Meats stored under sanitary conditions and refrigeration, have a shelf-life of 57 d without any increase in microbial contamination (*54*). However, *L. monocytogenes* can grow in refrigerated (4.4°C) processed meats. Growth is dependent on pH (well ≡pH 6 and not well ≡pH 5) and product type (*106*).

Bacterial Attachment and Biofilm Formation

Contamination of RTE meats is possible from the biofilms formed on food contact surfaces. Biofilms will form in five stages; nutrient transport, reversible adsorption step,

primary adhesion of microorganisms to surface, bacterial metabolism and colonization, and detachment (*55, 307*). Quorum sensing and cell-to-cell signaling, affect cell attachment and detachment of biofilms (*72*). Water channels, a convective flow, exist in biofilms to deliver nutrients to and remove waste from the microcolonial niche (*51*). Advantages of being in biofilms include protection from environment (ultra-violet radiation), as a trap to acquire nutrients, and resistance to antimicrobial agents, heat, and bacteriophages. Disadvantages are energy losses, reduced flow and heat transmission, and blocked membrane pores (*38, 227*). Detachment of biofilms is characterized by shearing or sloughing. Shearing continuously removes small portions while sloughing randomly removes massive portions due to nutrient or oxygen fluctuations within the biofilm (*262*). Detachment can occur from sonication or sequential application of sodium hydroxide (NaOH) and acetic acid at 55°C for 5 min for *L. monocytogenes* (*11, 17*).

Factors affecting attachment and biofilm formation include cells grown at optimum conditions (30°C, pH 7.0), pH, strains, its persistence in the plant, cell numbers, time, temperature, and nutrient type (*143, 171, 263, 264*). Optimal conditions for *Listeria* attachment are growth at 30°C and pH 4.76 (*75*). Major forces of attraction and repulsion between cells and surfaces are long range van der Waals interactions and short range hydrogen bonds (*174*).

Biofilm or glycocalyx production is a complex colony of single or multiple types of microorganisms embedded in a matrix of organic polymers found in marine environments, water pipelines, heat exchangers, cooling towers, implanted medical devices, food fermentation, and wastewater treatment (*38, 55, 135, 262, 302, 307*). After cleaning, survivors form biofilms on irregular or difficult to clean surfaces and re-grow. They then

become more resistant to sanitizers and heat, thus capable to contaminate foods (*26, 83, 302*). Cells embedded in biofilms receive less nutrients and oxygen becoming anaerobic, altering physiological properties and decreasing growth rate to sustain viability. However, with an increase in nutrients, biofilm thickness and resistance to antimicrobials increased. *L. monocytogenes* cells attached 8 d are 100 times more resistant to hypochlorite than those for 4 d. Size of biofilm depends on age and strain type (*28, 38, 47, 138, 155, 215*).

Pure cultures of *L. monocytogenes* do not form extensive extracellular polymeric substance (EPS) that mediate cell and substratum to colonize surfaces. Strains attach to stainless steel as single cells. Extended biofilms are formed in the presence of *Pseudomonas fragi*, a psychrotroph found in refrigeration temperatures. *P. fragi* produce EPS constituting 50 to 90% of total organic carbon in biofilms. Listerial cells are trapped and embedded in this EPS layer which act as a water-laden gel protecting cells from desiccation, incorporating water by hydrogen bonding, and can be both hydrophilic and hydrophobic. *Listeria* in biofilms is more resistant to sanitizers as the sessile cells reduce accessibility of sanitizers (*53, 72, 117, 135, 138, 156, 250*).

Attachment to Surfaces. Initial surface attachment is affected by hydrophobicity and hydrophilicity (*55, 138, 293*). The order (highest to lowest) of attachment of *L. monocytogenes* to stainless steel by serotype is 1/2c, 4b, and 1/2a (*215*). Attachment depends on initial cell numbers, bacterial species, and type of surfaces (*50*). *L. monocytogenes* attaches to surfaces in 20 min and 1 h at ambient and cold temperatures, respectively, possibly from flagella formation at 22°C and none at 37°C, which may affect attachment at different temperatures (*175, 293*). Exopolymer production is more important than

hydrophocity, surface charges, and flagellar formation in attachment of *L. monocytogenes* (*69, 250*).

Hydrophilic and high negative charged surfaces include glass, metals, stainless steel, and mineral where Gram-negatives attach. Hydrophobic and low positive or negative charged materials are plastics, Teflon®, and rubber. Higher energy (positive or negative) surfaces readily absorb solutes or nutrients enhancing bacterial colonization (*58, 250, 262, 263*). Attachment to surfaces is affected by time and if cells are in the reversible (weak interactions between bacteria and surface and removed by rinsing) or irreversible (formation of extracellular material and removed by stomaching) stage (*17, 297*). The reversible stage is affected by cell surface factors like EPS, flagella, and surface charge (*69*). Stainless steel surface contains grooves and crevices while glass is smoother but has scratches and depressions where bacteria are found (*175*). Upon exposure to sanitizer or heat, *L. monocytogenes* cells attached to glass survive 10 times longer than planktonic cells (*215*). *L. monocytogenes* attaches to stainless steel, Teflon®, nylon, and polyester floor sealant as a single species biofilm but less readily to cast iron drains (*22, 262, 270*).

Cell Surface Charge, Hydrophobicity, and Contact Angle Measurement (CAM). Adhesion of bacteria is more extensive to hydrophobic surfaces of low surface energy. Bacterial cells and some surfaces have net negative charges (capsules, lipopolysaccharides, proteins) producing strong electrostatic repulsion force, which is penetrated by cell fibrils and initiates surface attachment. Initial microbial deposition is impeded by electrostatic repulsion. High ionic strengths and low pH lowers the energy barrier (*26, 174, 175, 270*). Hydrophilic uncharged surfaces give the greatest resistance to protein adsorption, indicating less attachment (*55*).

Bacteria cell wall constituents such as phosphate, carboxylate groups, and proteins contribute to charges (*31*). The magnitude of this negative charge varies with strains (*69*). Greater net negative surface charge positively correlates to attachment to beef tissue surfaces (*68*). However, rough strains of *L. monocytogenes* lacking specific cell wall proteins, have greater surface charge but do not attach readily to beef (*68*). Charge and electron donor and acceptor properties influence attachment to surfaces. *L. monocytogenes* has a high negative charge with an electrophoretic mobility at -2.07 μm/V/cm/s from the low pKa (<2.1) of phosphate groups in phosphodiester bridges of cell wall teichoic acids (*31, 68, 174, 224*). A higher cell surface negative charge increases attachment to lean or fat meat surfaces (*71*). Electrostatic interactions and hydrophobicity are inversely related (*157, 289*). As hydrophobicity decreases, electrophoretic mobility increases attachment (*71*).

Cell surface hydrophobicity is affected by growth temperature, pH, time, and bacterial growth and concentration (*157, 289*). Cells cultured at faster growth rates are more hydrophobic (*289*). Developed methods to determine hydrophobicity include hydrophobic interaction chromatography (HIC) and contact angle measurements (CAM). Hydrophobicity increases as pH decreases, with age, and correlates well with attachment to fat tissue only (*15, 26, 58, 71, 81, 157, 174, 196, 240, 265, 272*). Clustering of nonpolar bonds of amino acid side chains causes hydrophobic bonds between cells and surface (*75*). Hydrophobicity is linearly related to attachment but not correlated with the numbers of attached bacteria. This indicates that attachment is affected by cell surface characteristics and not by the number of adherent cells (*15*). *L. monocytogenes* is hydrophilic based on physiochemical characterization and CAM (*174*). *L. monocytogenes* stored at 4°C is more hydrophobic and adhere more to stainless steel than the ones stored at –80°C (*30*). Adipose and lean meat

tissues show no difference in attachment. Bacteria on adipose tissue are harder to wash off due to the hydrophobicity (*50*). Lean meat tissues treated with electrical current as a meat tenderizer via the positive terminal, increase the total number of cells attached (*69*).

Contact angles are measured by dropping an aqueous solution (water or buffer) onto a solid substrata surface or microbial lawn and the 'angle' is measured from close-up slides of drops taken from a camera (*26, 123, 133*). The angles are defined in relation to surface free energies in equilibrium according to the Young equation:

$$\gamma_{lv} \cos \theta = \gamma_{sv} - \gamma_{sl}$$, where l is liquid, v is vapor, and s is solid.

This method differentiates microbial strains based on angles on microbial lawns, which quantitatively measures 'intrinsic microbial cell surface hydrophobicity' (*26, 239*). Results indicate that *L. monocytognes* has a free surface energy of 66.3 mJ/m^2 with an angle of 26.3° with water (*174*).

Cationized Ferritin. Treatment of bacterial cells with cationized ferritin (CF) stain followed by glutaraldehyde fixation and examination under scanning electron microscope (SEM) provides topographical views of cell surface. As one of the principal forms of iron storage in organisms, CF increases microscopic resolution of cell surface protuberances and only labels negatively charged surface structures. Glutaraldehyde inactivates cells and maintains structure (*10, 23, 26, 150*). However, cells during rapid growth have a positive surface charge repelling CF, hence, on slow growing cells, more protuberances are observed (*23*). Previous studies and improved methods include bacterial capsule, cell wall, and S-layer using transmission electron microscopy (TEM) and scanning electron microscope (SEM) (*10, 303*). *L. monocytogenes* forms extracellular fibrous-like materials, perhaps condensed EPS, between cells for attachment (*138, 210*).

Post-processing Contamination

Contamination of foods by *L. monocytogenes* occurs primarily after cooking. Pre-processing conditions may be important in transferring *Listeria* to sausages, such as from grinders and stuffers due to biofilm formation on the equipment (*57*). However, cooking solves this problem. *Listeria* spp. is found in liquid exudate or on the package but not in the internal meat in retail frankfurters, indicating post-process contamination from peeling and packaging (*20, 126, 220*). In a plant manufacturing turkey frankfurter, the peeler is found to be a major source of contamination after cooking but prior to packaging (*116*). After heat processing, retail slicing increases bacterial contamination (*8, 124*). Dicing machine also retains and transfers contaminants (*170*). Higher incidence rates are obtained from whole cooked meat products after slicing (6.65%) than before slicing (1.56%), indicating cross-contamination (*288*).

Irradiation

The World Health Organization (WHO) has approved food irradiation and the technology is used in 35 countries, mainly to decontaminate spices (*169*). Food irradiation in the United States has been used in space programs, commercial applications, medical products, and therapeutic diets for immunocompromised persons. Current approvals include beef, pork, and poultry and this technology has been applied to poultry and poultry products, red meats (hamburger), dried herbs and spices, seafood, fruits and vegetables, bulbs and tubers, cereal grains, and ready meals (*21, 64, 129, 151*). Irradiation preserves perishable foods to extend shelf-life, reduces chemical preservatives, disinfects insects, inhibits sprout, and reduces post-processing contamination. Irradiation is effective against pathogens by

breaking the double stranded DNA (*25, 99, 140, 145, 193, 195, 230, 275*). Indirect effects include production of free radicals damaging cellular constituents (*127*).

High dose (>10 kGy) irradiation produces commercially sterilized food products. Doses of <10 kGy reduce viable populations to extend shelf-life (*194*). Doses ≤10 kGy have no toxicological hazards in foods (*180*). Many studies have been conducted with fresh, cooked, and processed meats (*99, 108, 113, 127, 149, 153, 180, 188, 193, 253, 276*). Irradiation processes to decrease 3 to 5 $\log_{10}$ of *L. monocytogenes* help increase refrigeration storage time 2 to 4 weeks (*149*).

The effectiveness of irradiation and resistance of microorganisms is dependent on growth phase, types and numbers of microorganisms, pH, temperature, chemical composition and preservation of foods, product formulation, and atmospheric gas composition (*180, 191, 194, 268*). Oxygen increases irradiation lethality on bacteria, but adversely affects organoleptic qualities (*149*). Irradiation sensitivity is Gram-negative > Gram-positive > yeast > mold > fungal spores > aerobic and anaerobic spore formers. Vegetative cells are more sensitive because of free radicals from intracellular water (*180*). Irradiation increases the lag phase duration of microorganisms, slows growth, increases shelf-life, and perhaps decreases virulence but alters residual microbial population (*222, 223*).

Approved irradiation sources are gamma, electron beam, x-ray, microwave, and ultraviolet energy (*119, 191, 304*). Radiation sensitivity, expressed as D_{10} values, is defined as the dose required to reduce a population by 90% (1 $\log_{10}$), and is calculated as the absolute value of the reciprocal of the slope of the thermal death time (TDT) survival curve (*167*). Reported D_{10} values for *L. monocytogenes* range from 0.2 to 2.0 kGy depending on strain,

substrate, and conditions (*100, 266*). Average D_{10} value for *L. monocytogenes* on frankfurters is 0.61 kGy using gamma irradiation (*268*).

Irradiated ground beef is currently available in grocery stores in United States (*200, 204, 206-208, 248*). Restaurants are offering irradiated hamburgers on their menus (*185, 246*). Irradiated frozen ground beef patties, boneless chicken breasts, and chicken tenders will be commercially available in 2003 (*209*). Consumer acceptance of food irradiation can be enhanced with good communication strategies to provide proper information and to address public concerns (*32*). Acceptance is more positive with more publicity in the recent application in anthrax-contaminated mail (*64*). Irradiated strawberries, oranges, and grapefruits outsold nonirradiated ones at 9:1 ratio in test markets (*129*). Consumer concerns include food with reduced nutrients or which are radioactive, and as a replacement of good hygienic practices in processing plants, must be addressed (*91, 129, 142, 180*). Currently, irradiated foods and those containing a significant portion of irradiated food, must be labeled (*21*).

Sensory Qualities

Although irradiation and cool storage effectively extend shelf-life by controlling microbial growth, changes in sensory characteristics limit use. Such changes include flavor, odor, color, texture, appearance, and nutrient quality making them unacceptable, in some cases, for consumption (*108, 109, 129, 183, 194, 255*). Irradiated foods contain the same nutrient quality as those preserved by canning, freezing, and drying. Organoleptic changes can be minimized using low doses (≤3.5 kGy) to eliminate pathogens. However, at low

doses, small numbers of bacteria may survive and recover from sublethal injury, and then multiply to detectable levels.

Preserving positive organoleptic qualities requires products to be frozen and vacuum-packed to reduce oxidative rancidity generated during irradiation (*140, 151, 188, 193, 195, 255, 268, 276*). Organoleptic quality of irradiated ham without sodium nitrite is acceptable (*36*). Freezing meat may cause off-flavors from oxidation of polyunsaturated fatty acids, which can be reduced with proper packaging (*49*). Meat irradiation in the presence of oxygen accelerates lipid auto-oxidation resulting in off-flavor or odor (*195*). Irradiated hams have burnt and sulfurous off-flavors and off-odors. Reduction of these undesirable odors must be considered with irradiation processing (*115*). Irradiation does not alter pH, color, and odor for hams but changes thiobarbituric acid (TBA as a measure of fat rancidity) in bologna sausage and color in pork loin (*37, 99, 153*). Irradiated cooked beef at 3 kGy is tougher and more chewy than nonirradiated beef and shows a distinct pink color from metmyoglobin reduction which will be re-oxidized to metmyoglobin in oxygen during storage (*183*). In conducting sensory evaluation, the order of sample presentation is important, as a natural bias occurs for the first sample tasted (*115*). Therefore, random presentation of samples must be conducted to obtain unbiased data (*152*).

Prevention and Treatment of *L. monocytogenes*

The United States Department of Agriculture (USDA) implements a zero tolerance standard of *Listeria* in ready-to-eat (RTE) meats but is not practical as these are not sterilized products (*169, 178, 202*). Guidelines to prevent and control contamination by *L. monocytogenes* are documented (*279, 281*). The Danish believe 'zero-tolerance' is

unrealistic, leading to unnecessary recalls of wholesome foods and loss of consumer confidence. The primary issue is to control survival and growth rather than prevention. International Commission on Microbiological Specifications for Foods (ICMSF) recommends that in RTE foods for people not at risk, the level of *L. monocytogenes* be less than 100 cells/g at time of consumption and for high-risk groups, absence in 25 g (*84, 228, 278, 291*). Cook-chill and -freeze catering systems in Britain require no *L. monocytogenes* be detected in 25 g of food at the end of chill storage, reheated to a core temperature of 70°C for 2 min, and reheated food served within 15 min (*301*).

Effective cleaning and sanitizing procedures for equipment and plant minimize microbial contamination and biofilm formation (*307*). Effective compounds against *L. monocytogenes* include iodophors (25 ppm iodine), alkaline sanitizers, acid sanitizers (130 to 200 ppm peracetic or peroctanoic), chlorine [20 ppm for water treatment, 200 ppm for cleaning-in-place (CIP), stainless steel equipment, walls, and nonporous surfaces, and 1000 ppm for wooden crates, porous surfaces, and atmosphere fogging], and quaternary ammonium compounds (QAC) at 200 ppm (*83, 86, 210, 274*). Chlorine sensitivity depends on strain and the efficiency decreases with increasing pH, but chlorine is more effective at 5°C and 35°C than at 25°C. On stainless steel, QAC at 50 ppm for 1 min destroys *L. monocytogenes* (*100*). Hydrophilic cationic molecules, QAC, readily absorb, penetrate, and disrupt cytoplasmic cell membrane. Gram-negatives are more resistant to QAC due to lipid contents of the outer membrane and Gram-positives produce lipoteichoic acids (lipophilic) preventing penetration (*92*). Exposure to high concentrations of alkaline cleaning solution renders *L. monocytogenes* sensitive to subsequent sanitizers (*274*). Rotating sanitizers is more effective so that microorganisms do not become adapted to a particular sanitizer and

develop resistance (*280*). Susceptibility to disinfectants depends on the presence of organic material and a protective shield of biofilms (*83, 86*). Disinfectants for planktonic cells are different from those for the more resistant biofilms (*156*). Chemical shock, however, does not affect cell attachment to stainless steel (*226*).

The *Listeria* Performance Standard depends on types of product made, number of manufacturing lines, age of plant, and unique equipment or processes used (*161*). Processing plants implement improved or reassess Hazard Analysis and Critical Control Point (HACCP) principles 'from farm to fork', Pathogen Reduction, Good Hygienic Practice (GHP), and Good Manufacturing Practice (GMP) (*202, 228, 243, 244*). Critical control points for processed meats are cooking, chilling and storage temperatures, air, contamination prevention, and possibly irradiation. Recontamination can be minimized by separating raw from cooked foods by reducing human and forklift traffic, separating wet and dry processes, storing and packaging exposed product in clean and dry environments, and using properly designed and maintained equipment. Modification of processing procedures such as higher thermal treatment for better destruction, more frequent and thorough cleaning and sanitizing of equipment and environment, and temperature controls can be considered (*110, 116, 176*). Equipment design is essential; it should be free of recesses, open seams, gaps, protruding ledges, exposed bolts and rivets, and free of dead ends (*178*). Sanitary plant design and maintenance, and constant monitoring or sampling of processing environment facility, are critical (*116, 139, 244*).

Product reformulation, antimicrobial agents, reduced temperature (<2°C) storage and shelf-life, post-processing pasteurization of meat product (submersion heating), better distribution patterns, and consumer information need to be considered (*20, 106, 119, 198,*

203, 228, 285). Hurdle technology is a combination of substances or processes to prevent or inhibit microbial, chemical or physical deterioration of foods to improve safety, stability, and quality (*60, 105, 228*). High hydrostatic pressure (HHP), irradiation, high voltage electric discharges, high intensity light, raised pressure (manothermosonication), and mild heating with ultrasonication are alternative decontamination technologies (*169*). HHP transmits instant pressure damaging cell membrane, increasing permeability, and finally cell death with a tailing effect (*197, 242*).

Quantitative microbial risk assessment (QMRA) and predictive modeling attempt to improve food safety by examining available scientific data and information systematically to estimate relative risks of serious illness and death from foods that may be contaminated with *L. monocytogenes*. The QMRA provides information on hazard identification (disease, etiology, food category identification); exposure assessment (food consumption and contamination, post retail growth); hazard characterization or dose response (human susceptibility, virulence, food composition or matrix, dose-response model); and risk characterization. The USDA-ARS Pathogen Modeling Program (PMP) and models derived for intrinsic and extrinsic factors affecting microbial growth rate and lag time are available. Variability of growth among strains and food types or formulation may limit the usefulness of modeling (*13, 65, 85, 87, 216*). An action plan to reduce risk of foodborne listeriosis is also available (*87, 285, 286*).

Consumer education is imperative. Consumer knowledge of manufacturer's recommendations on proper storage, preparation, and expiration date, are important (*87*). All consumers are advised to consume perishable foods (RTE meats) as soon as possible, clean refrigerators regularly, and use a thermometer to ascertain the temperature remains <40°F

(*286*). Hot foods must be kept hot (>140°F) and cold foods cold (<40°F). Foods in shallow containers need to be chilled rapidly (*178*). High-risks groups should avoid foods with relatively high incidence of *L. monocytogenes* such as prepared salads and coleslaw; unpasteurized dairy products; uncooked, refrigerated, or smoked fish and shellfish; refrigerated patés and meat spreads; and soft cheeses. Caution must be taken when using microwave ovens to reheat foods. Microwave ovens produce cold spots from unequal energy distribution, which can cause improper heating of foods (*100, 146, 259*). Leftover foods and processed meats should be reheated to 160°F prior to consumption (*41, 212, 248, 285*). To keep food safe, the USDA recommends to clean hands and surfaces often, to separate foods (not cross-contaminate), to cook (proper internal temperature), and to chill foods promptly (*285*).

Environmental Contamination

Environmental contamination of *L. monocytogenes* in foods is low in population but hazardous. This pathogen resides on drainpipes, surfaces, and spiral threads of screws, potentially contaminating post-processed foods (*1, 73, 176, 213*). *Listeria monocytogenes* are found in meat plants on product contact surfaces (conveyor belts, transport containers, cutting tables, equipment and boards), air above fans and air conditioners, exhaust hoods, humidifiers, employees' clothing and hands, drains and gutters, pickling systems, and cleaning equipment (*14, 83*). Locations consistently harbor *Listeria* include floors (37%), drains (37%), cleaning aids (24%), wash areas (24%), sausage peelers (22%), and walls and ceilings (5%) (*178*). Contamination sites include door handles and seals; chiller shelving, walls, and doors; slicer and table; and scales (*110*). Floor drains are of concern from aerosol

formation during cleaning from using high-pressure hoses. If left unclean, nutritive waste materials allow *L. monocytogenes* to grow. Drains are suggested not to be located next to filling and packaging operations. High-pressure hoses are not recommended for cleaning. Perhaps a 5-gallon of 800 ppm of chlorine sanitizer can be used instead (*139, 270*).

Listeria monocytogenes is an environmental contaminant indicating contamination post-heating. As mentioned, the problem is aerosol formation from hoses used to clean floors. Aerosolization is an additional stress defined as 'the suspension of microscopic solid or liquid particles in air or gas' ranging from 0.5 to 50 μm. This organism (10^8 to 10^9 CFU/ml) survives in aerosol for 3.5 h. Condensate (7%) and compressed air (4%) are found to be positive for *Listeria* (*53, 84, 139, 178, 211, 270*). The factor that remains unknown is if *L. monocytogenes* is capable of surviving in dust or condensate in the environment. And if so, can this stressed bacterium from nutrient deprivation be able to come into contact with RTE meats and be able to repair and grow?

Outbreaks and Recalls

Numerous foodborne outbreaks due to *L. monocytogenes* have been documented. The 1981 Nova Scotia and 1983 Massachusetts outbreaks were caused by coleslaw made with cabbage contaminated with sheep manure and contaminated milk, respectively (*116, 210*). A 1985 California outbreak, serotype 4b in Mexican-style cheese caused 40 deaths and 100 illnesses (*3, 39, 287*). Turkey frankfurters were linked to human listeriosis when a cancer patient developed sepsis by serotype 1/2a in 1988 (*40, 146*). In France in 1993, human listeriosis occurred from consumption of porcine tongue jelly (*57, 132*). In 2002, the northeastern United States experienced an outbreak resulting in 10 deaths from sliced turkey

deli meat (*43, 46, 247*). Outbreaks have occurred from faulty food safety process, such as plant renovation, defective processing equipment, or inadequate pasteurization (*87*).

Much attention is focused on the *L. monocytogenes* serotype 4b multistate outbreak of 1998 to 1999, which resulted in 15 deaths, six miscarriages, and 101 illnesses. Government officials theorized that construction generated dust contaminated with *L. monocytogenes*, which later contaminated processing equipment and then food products. The plant was repairing and modernizing the air handling system during production. Thirty five million pounds of meat were recalled but only 6 million recovered. Some lawsuits were settled out of court, the company was charged with a federal misdemeanor, paid $200,000 in fines, and donated $3 million for food safety research (*1-6, 41, 42, 63, 95, 96, 103, 169, 199, 203*).

Processed meats which have been recalled due to *Listeria* contamination include hams, sausages, frankfurters, bologna, bratwurst, bauerwurst, meat loaf, processed beef, liver cheese wrapped in pork fat, turkey breast, chicken fajitas and salad, and kosher chicken chow mein entrees. Other non-meat recalls include salad shrimp and milk (*4, 95, 98, 103, 146, 158-165, 184, 205, 206, 245*).

Summary

The goal of this thesis is to better understand the ecology of *Listeria monocytogenes* in RTE processed meats, from the initial contamination of the meats to using irradiation as a form of intervention. Survival of *L. monocytogenes* in a condition simulating dust in the processing environment was studied using sand as a model. Once survival was ascertained, then perhaps *L. monocytogenes* in the sand was able to repair and recover and then contaminated selected RTE meats. An issue that was of concern was quantification of viable

but nonculturable (VBNC) cells that might not be recovered using the regular enumeration methods, i.e., plating on nonselective agar. The direct viable count (DVC) method was used to observe if *L. monocytogenes* become VBNC and if so, what sort of medium was most successful in providing the highest population recovery. While *L. monocytogenes* from dustlike vectors was found to be able to contaminate RTE meats, the mechanism that this bacterium used to attach to RTE meat surfaces was studied, especially the ones related to cell surface charge and cell surface hydrophobicity. Irradiation was studied as an intervention method. Low dose irradiation was examined and also possible survival and recovery of *L. monocytogenes* from meats irradiated at such doses. With this low dose of irradiation, a sensory evaluation was conducted to see if consumers could tell a difference between irradiated and nonirradiated selected RTE meats.

Much of this work is done by the author in collaboration with another graduate student. This paper has been accepted for publication in the Journal of Food Protection (*63*).

Introduction

Listeria monocytogenes contaminated processed meats has been incriminated as a source of foodborne outbreaks. Since processing of ready-to-eat (RTE) meats include high temperature cooking and under proper storage conditions, the product should be safe to consume. Contamination of *Listeria* occurs during post-processing and handling of these products as this bacterium is very fastidious and often found in the environment. Possible sources of this environmental contamination can be the physical environment, which include dust, airborne moisture, or condensate and the process itself which may be direct contact with

processing equipment or packaging films. The ability of *L. monocytogenes* to adhere to a multitude of these surfaces has been proposed as possible sources of contamination.

One notable outbreak of 1998 to 1999 resulted in 21 fatalities and approximately 100 reported cases of listeriosis (*42*). Centers for Disease Control and Prevention (CDC) officials cited construction in the processing plant as a likely source of the contamination. Inspectors theorized that dust from construction served as a vector for *L. monocytogenes* transmission onto the RTE meats post processing, but prior to packaging. At that time, the plant's air handling system was being upgraded enhancing the risk of airborne particulates contacting the product and or product contact surfaces (*1*).

Little scientific evidence exists that definitively describes or characterizes the ability of *L. monocytogenes* to endure stress conditions associated with being transported onto a ready-to-eat product by a dustlike vector. This research was designed to test the ability of *L. monocytogenes* to endure such stress and characterize its ability to contaminate ready-to-eat meat products when transported on a particulate vector similar to dust.

Materials and Methods

A five-strain mixed culture of *Listeria monocytogenes*: Scott A, 1/2a H7764, 4b H7969, 4b H7962, and OB90393, was used. Centrifuged cells were resuspended in Butterfield's phosphate buffer solution and mixed well with sterile sand which was divided into four portions. Groups or containers A and B were subjected to 10°C simulating the temperature found in the packaging area of processing facilities. Group A was subjected to 88% RH while group B was stored with a desiccant (RH below 10%). Groups C and D were

exposed to 22°C, with C at 40% RH while group D was desiccated. The experiment was conducted three times.

Four types of commercially available RTE meats were selected: frankfurters, light bologna, chopped ham, and roast beef. The meats were irradiated to eliminate the naturally occurring microflora and stored at 4°C until use.

Inoculation of the RTE meats was carried out every two days for the first eight sampling days, after which, inoculation was carried out every three days for the remaining nine sampling days, with a total of 17 sampling days. Each study took a total of 41 days (beginning at day 0). Irradiated RTE meats were placed individually into filter bags. Each of the four meat types was prepared in triplicates to be inoculated with sand vectors from each of the four sand treatment conditions. Bags were inoculated with 0.5 g of sand from the corresponding treatment code, vacuum-sealed, and allowed time for attachment. Diluent (Butterfield's phosphate solution) was placed into each meat/bag and homogenized. Half a gram of sand was placed into a buffer solution homogenized. Both meat and sand samples were plated onto tryptic soy agar with 0.6% yeast extract (TSAYE), modified Oxford (MOX) agar, and tryptic soy agar with 0.6% yeast extract plus 5% NaCl (TSAYE + 5% NaCl) in duplicates. Plates were incubated at 35°C for 24 to 48 h and then counted.

A daily survival rate was estimated for each replicate of each treatment using linear regression of $\log_{10}$ CFU/ml of *L. monocytogenes* against time in days. The estimated slope in this regression was the $\log_{10}$ CFU of the daily survival rate; more negative slopes corresponded to a lower daily survival. The estimated Y-intercept described the initial recovery of *L. monocytogenes*. Smaller values corresponded to less recovery of live cells on day 0. Treatments were also compared by computing the number of days until the predicted

concentration drops to 1 CFU/ml. That "effective survival time" was computed from the linear regression.

Results and Discussion

L. monocytogenes at 10°C (groups A and B) survived longer, thus capable to contaminate RTE meats longer than those stored at 22°C (groups C and D). Less injury occurred at 10°C. *L. monocytogenes* survived longer at 88% RH than under desiccation. At 22°C, the desiccated group D persisted longer and contaminated RTE meat products longer than the non-desiccated group C. Projected survival of *L. monocytogenes* in days were 151 days for group A (10°C, 88% RH), 136 days for B (10°C, desiccated), 73 days for C (22°C, 40% RH), and 82 days for D (22°C, desiccated).

The highest recovered populations of *L. monocytogenes* from the sand vector and meats came from the nonselective TSAYE for groups B to D. The daily survival rate was highest for TSAYE, followed by MOX and then TSAYE + 5% NaCl.

On sand vector, group A was expected to be the most favorable as the low temperature slows the cells' metabolism by prolonging the onset of starvation or dehydration of the bacteria while the high RH slowed the process of physical damage to the cells due to rapid drying. However, selective media were more successful in recovery than TSAYE. This finding was contradictory as TSAYE contained no selective agents and most cells should recover. An explanation was that the base medium of MOX was Columbia agar which was more nutritious than TSAYE, hence showed better recovery.

Group A (10°C, 88% RH) yielded the highest populations of recovered cells and had the highest daily survival rate. Group B (10°C, desiccated) produced more injury and

resulted in a more rapid decrease in detectability. Group C (22°C, 40% RH) simulated conditions in non-refrigerated areas within a processing facility. Cells would utilize their nutrient stores faster at 22°C and enter a state of injury more rapidly than those cells stored at 10°C. Refrigerated but moist environment resulted in greater survival of *L. monocytogenes* compared to treatment D (22°C, desiccated) which yielded the lowest populations. Elevated temperature with desiccation caused the most injury to the cells.

The four RTE meats behaved similarly. An increase in population recovery occurred with <1.5 $\log_{10}$ CFU/ml greater on the meats than those on the sand vectors. This difference increased over time. *L. monocytogenes* from the sand vectors can repair from environmental stress and contaminate RTE meats. Frankfurters were most favorable for growth while roast beef was the least. Addition of inhibitory ingredients inhibited cellular repair. *L. monocytogenes* from the vector inoculated onto RTE meats could repair and divide.

Conclusions of this Study

Recent outbreaks of listeriosis associated with RTE processed meats have been attributed to environmental contamination. This situation requires a scientific explanation off the feasibility of airborne particulate matter such as dust carrying *L. monocytogenes* being a potential source of contamination. Data from this experiment indicate that *L. monocytogenes* could survive both desiccated and nutrient depleted on the surface of a dustlike vector and could then contaminate RTE meat.

The complex composition of dust in a single location varies from day to day depending on a large number of factors and thus would be a difficult model for scientific study. The sand model used here is a repeatable and controllable system. No nutrients are

available on the sand used as a vector, and the sand is capable of being desiccated quickly and effectively. Dust in a food-processing area has much more potential to harbor nutrients and moisture resulting from its constituents and environment and therefore to serve as a wholesome and protective environment for bacteria.

The data obtained indicate that *L. monocytogenes* is capable of enduring a dustlike environment and contaminating RTE meats better under refrigerated, high-relative-humidity conditions similar to those in a RTE meat-processing environment. Drying plays a role in limiting the survival of the pathogen over time but does not limit its ability to repair and flourish once it encounters a favorable environment, as seen for groups C and D. Temperature plays a role in limiting the survival of *L. monocytogenes* on a dustlike vector over time. *L. monocytogenes* from groups held at 10°C were found to survive longer and to contaminate RTE meats longer than those held at 22°C. This finding may be attributed to differences in metabolic activity. More research is necessary to explain the metabolic activity of cells placed under this type of stress and the ability of the bacterium to repair itself under conditions associated with RTE meats.

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CHAPTER 2.

SURVIVAL AND RECOVERY OF VIABLE BUT NONCULTURABLE (VBNC) *LISTERIA MONOCYTOGENES* CELLS IN A NUTRITIONALLY DEPLETED MEDIUM

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ABSTRACT

Survival of a five-strain *Listeria monocytogenes* culture during storage in sand at 4°C for 2 months was determined using the acridine orange direct count (AODC) method using novobiocin and plate count. Samples previously inoculated with *L. monocytogenes* were taken every two weeks. The inoculated sand was then subjected to a 6-h incubation at 37°C and stained with acridine orange. Elongated VBNC cells were observed more often during weeks 2 and 4 under a fluorescent microscope. At weeks 6 and 8, most of the cells either remained viable or were dead. In each microscopic field, only one or two VBNC cell was observed among hundreds of other viable culturable cells, indicating that *L. monocytogenes* does not generally become VBNC. Either the bacterium is viable and culturable or dead. Therefore, when plating environmental samples or desiccated *L. monocytogenes* cells on nonselective media, VBNC cells are not a concern. Media used for plate count include tryptic soy agar with 0.6% yeast extract (TSAYE) and Columbia agar as nonselective media and modified Oxford (MOX) agar and TSAYE + 5% sodium chloride as the selective media.

Variations within these media were aerobic or anaerobic incubation and with 0.1% or 1% sodium pyruvate to determine the best condition to recover the highest number of injured cells. Results from the nonselective media showed better recovery observed on TSAYE with 0.1% pyruvate incubated aerobically and Columbia agar with 0.1% pyruvate incubated aerobically. However, these methods were comparable with each other ($P > 0.05$) in recovering desiccated *L. monocytogenes* cells.

INTRODUCTION

Bacteria in natural environments are exposed to nutrient fluxes, and in order to survive, they must adapt to hostile environments leading to the development of strains that show increased resistance to normal levels of homologous or heterologous (cross-protection) inimical stresses (*13, 16, 29*). In the environment, *Listeria monocytogenes* can be stressed structurally or metabolically by sanitizers (amines, quaternary ammonium compounds, and peroxides), preservatives, heat, cold or freeze, dry, osmotic, alkaline, and acid (*1, 5, 6*). Compared to healthy cells, fewer numbers of starved cells were found to attach to beef (*10*). Stressed or injured cells are difficult to enumerate using regular direct enumeration procedures. Some cells remain viable but are unable to grow on media, which results in underestimating surviving population and overestimating processing efficacy. This 'bacterial cell suicide' explains 'viable but nonculturable (VBNC)' state where stressed cells show normal respiratory and metabolic functions but cannot be cultured on standard media (*29*). The rate of decrease of cell viability is far less than the rate of culturability (*7, 19*). This VBNC state has been observed in human pathogens such as *Escherichia coli*, *Salmonella enteritidis*, *Vibrio* spp., *Legionella pneumophila*, *Staphylococcus aureus*, *Bacillus subtilis*,

Campylobacter jejuni, *Listeria monocytogenes*, and *Shigella* spp. (*2, 8, 20, 21, 26, 27, 29*). The rate for nonculturable response was affected by nutrient deprivation, temperature [intermediate (25 to 37°C) faster than lower (4 to 5°C)], sodium concentration, growth phase, and use of disinfectants (*3*). Substrates incorporated at this stage are used for maintenance functions and not for growth (*27*). Some physiological and morphological changes occur at this state during nutrient deprivation, such as, reduced metabolic rate and decrease in cell size due to depletion of cellular reserves, hence, minimizing maintenance requirements, i.e., for respiration purposes (*14, 28*).

A direct microscopic method was developed to detect these VBNC cells (*15, 18*). This direct technique was initially used in marine microbiology to test water samples but is now applied to studying environmental contaminants which are often stressed (*12, 18*). With this technique, viable and culturable, VBNC, and dead cells can be differentiated. Viable cells are metabolically active and can be detected using normal enumeration methods. The VBNC cells are metabolically active but cannot be detected using normal enumeration methods. Dead cells are metabolically inactive thus will not be detected using plating methods.

L. monocytogenes was observed to have lost culturability but remained viable after 10 weeks under starvation in water (*3*). A sand model, slightly modified from De Roin et al. (2002), was used in this experiment to mimic survival of *L. monocytogenes* in a dustlike environment. More counts (at least half a log) were observed using this direct viable count (DVC) method compared to recovery from nonselective media and even more so than selective media (*12, 29*). This study was undertaken to determine if *L. monocytogenes* goes into VBNC state, how long it remains at that state under induced stress, and if it proves

important in detection using standard plating media. Different kinds of media and modifications were investigated to determine the best method to recover most stressed cells.

MATERIALS AND METHODS

Bacterial cultures and growth conditions. Five individual strains and a mixed cocktail of the five strains of *Listeria monocytogenes* [Scott A (FSRL culture collection), 1/2a H7764, 4b H7969, 4b H7962, and 4b OB90393] were used in this experiment. All strains were grown and maintained in tryptic soy slant with 0.6% (wt/vol) yeast extract (TSAYE; Difco Laboratories, Detroit, MI) at 4°C until needed. Each strain was cultured independently in 10 ml tryptic soy broth with 0.6% (wt/vol) yeast extract (TSBYE; Difco, Becton Dickinson, Sparks, MD) at 37°C for 24 h to revive the cells. A mixed culture was obtained by inoculating a 10 ml tube of TSBYE with 200 µl of each of the individual strains. One milliliter from this mixed culture was transferred to 500 ml of TSBYE in a pyrex bottle followed by incubation at 35.5°C with agitation for 14 h (Lab Line, New Brunswick, NJ). Cells were harvested at late log phase by centrifugation using the Sorvall® Super T-21 (Kendro Laboratory Products, Newton, CT) at 2,000 x g for 20 min at 5°C. The pellet was washed twice with 250 ml of Butterfield's phosphate solution (BPS) and resuspended in 50 ml BPS. This was considered as the inoculum for the sand. Cell count of the resuspended cells in the 50 ml of BPS was determined by plating in duplicate samples onto TSAYE and modified Oxford (MOX) agar (Difco Laboratories, Inc., Detroit, MI). The entire experiment was independently replicated three times.

Sand preparation and cell starvation. White quartz sand (-50 +70 mesh size) was obtained from Sigma (St. Louis, MO). Two hundred and fifty grams of this sand was

autoclaved twice in a beaker. The mixed culture of *Listeria monocytogenes* inoculum and the sand were mixed in a sterile Corningware dish in the laminar flow hood. The sand was covered and left to dry for 24 h prior to transferring it to a sterile (UV treated) labeled Rubbermaid (Wooster, OH) storage container. This container was then stored at 4°C for 2 months.

Direct Viable Count (DVC). One gram of sand was transferred to 3 ml of BPS and mixed. Double strength TSB with 1.2% yeast extract (3 ml) and 1 ml of novobiocin (30 mg/ml; Sigma, St. Louis, MO) were added to the gram of sand and mixed. Previously reported concentration of novobiocin was 28 μg/ml but this was found to be too low to show distinctive VBNC cells (*12*). Incremental concentrations were used, i.e., 30 μg/ml, 300 μg/ml, 3 mg/ml and 30 mg/ml, and the most distinctive VBNC cells were found using the highest concentration of novobiocin (unpublished data). The tube containing sand and novobiocin media was incubated at 35°C for 6 h, after which 1 ml of the mixed solution was taken and transferred to 5 ml of BPS. This 6 ml was then filtered through a black 47 mm 0.22 μm Nuclepore® Polycarbonate Track Etch Membrane (Whatman Inc., Clifton, NJ) using the Nalgene Vacuum Manifold filtering system (Fisher Scientific, Springfield, NJ). The filter was fixed with sterile 95% ethanol for 2 min, drained, and allowed to air dry for 2 min. Acridine orange [0.01% (wt/vol); 1 ml; Sigma, St. Louis, MO] was added to the filter, allowed 5 min to stain, and then drained. The filter was washed twice with 5 ml of sterile distilled water and placed in a sterile petri plate to dry. Cells fixed on the filter were observed under epifluorescent microscope at 100x and the microscopic fields were photographed using the AxioVision 3.0.6 SPA software. Viable and culturable cells would be fluorescing orange and be regular size. Viable but nonculturable cells would be

fluorescing orange also but of elongated size. Cells that are at least twice the length of viable and culturable cells were determined to be VBNC. Dead cells will be fluorescing green or white.

Conventional plate counts. Sand samples were taken from the container at two-week intervals for 8 weeks. One gram of sand was aseptically transferred to 9.9 ml BPS and serially diluted. Viable cell populations were enumerated on nonselective and selective media according to Table 2.1. The pyruvate used was a sodium salt of pyruvic acid (α-keto-propionic acid; Sigma, St. Louis, MO). Plating was carried out using the DW Scientific Whitley Automatic Spiral Plater (West Yorkshire, England). Plates were incubated at 35°C for 48 h, and colonies were counted using the Sypnosis Ltd. (UK) ProtoCOL (model 60000) automated plate counter.

RESULTS AND DISCUSSION

Direct Viable Count. Stressed cells are able to replicate a limited number of times before entering a 'dormantlike' state. However, the longer the time the cells were in a dormant state, the more complex conditions were required for the cells to resume growth and division (*28*). After being exposed to stress, injured cells required time to repair and recover. During this period, cells synthesized membrane lipids, phospholipids, protein, ATP, ribosomal RNA, and repaired breaks in single stranded DNA (*11*).

In this experiment, samples pre-incubated with yeast extract to promote growth and a DNA synthesis inhibitor prevented cell division but allowed other synthetic pathways to proceed which resulted in elongated cells (*18, 23, 27*). Some examples of DNA gyrase inhibitors to prevent DNA replication include nalidixic acid for Gram-negatives and

novobiocin, ciprofloxacin, enrofloxacin, enoxacin, norfloxacin, and isopropyl cinodine for Gram-positives (*2, 3, 12, 18, 23, 29*). The samples were filtered through nuclepore filters and stained with acridine orange (*15, 18*). Nuclepore filters were preferred as higher counts were obtained compared to cellulose filters (*4*).

The different kinds of cell viabilities observed using novobiocin under fluorescent microscope are shown in Figure 2.1. Acridine orange stain will bind to all viable cells containing abundance of cellular RNA causing them to fluoresce reddish orange under a fluorescent microscope. Viable and culturable cells were much shorter in length compared to the VBNC cells. The VBNC cells were much longer (at least two to three and up to 10 times) than the viable and culturable ones. Cell elongation for *L. monocytogenes* increased from 0.8 to 1.5 μm to a length of 2.4 to 4.8 μm (*12*). Dead cells showing a complete loss of viability with their low concentration of RNA will fluoresce greenish white (*18*). The incubation time and type and concentration of the inhibitor used are important in determining the maximum number of VBNC cells (*2*). The progression of stress-induced *L. monocytogenes* over the 2-month period is shown in Figure 2.1. Initially at week 0 when the cells were being harvested, very few VBNC cells were observed. In each microscopic field, only one or two cells were found to enter this state among hundreds of other viable and culturable cells. In weeks 2 and 4, more (two to three) VBNC cells were found with more frequency shifting through microscopic fields. During the last four weeks (6 and 8), finding any VBNC cells was difficult. Cells either remained viable and culturable or dead. Also cells were observed to exist in two cells attached together, in clumps, or in chains.

Conventional plate counts. A comparison of populations enumerated on the various media (nonselective and selective with TSAYE as the reference) in the recovery of stressed

induced *L. monocytogenes* is shown in Figure 2.2. Previous data indicated that higher populations were recovered on Columbia agar than TSAYE (*9*). Results from the present experiment did not agree with this. Cells recovered from both types of media were comparable to each other ($P > 0.05$). Previous studies showed anaerobic incubation to have better recovery of heat-injured *L. monocytogenes* (*17, 24*). However, results from this experiment showed the contrary, that is, lower counts (<1 $\log_{10}$ CFU/ml) were obtained with anaerobic compared to aerobic incubation. This result agrees with Romick et al. (1996) who found the maximum cell density of anaerobic culture was 1 $\log_{10}$ unit lower than the one for aerobic culture. This difference may be attributed to the types of injury caused by heat compared to desiccation.

Sublethally-stressed bacteria were shown to be sensitive to oxygen tension, thus better recovery could be obtained by plating them on minimal rather than rich media. Additions of oxygen scavengers, for example sodium pyruvate, glutathione, or Oxyrase®, to minimal media, assist recovery of stress-injured cells (*29*). Pyruvate also served as a primary substrate in the Krebs Cycle to generate energy for growth. Even though minimal media were not used, sodium pyruvate was added to TSAYE, Columbia agar, and MOX at 0.1 and 1%. Adding 0.1% pyruvate gave similar results to the same media incubated aerobically. When 1% pyruvate was added, the recovery was much lower (<1 $\log_{10}$ CFU/ml) than with 0.1%.

The nonselective media with the highest recovered population of stress-induced *L. monocytogenes* are shown in Figure 2.3. These were TSAYE and Columbia agar incubated aerobically and with addition of 0.1% pyruvate. No significant difference ($P > 0.05$) was found. Higher recoveries were visually observed on the nonselective media compared to the

selective media. This was common as some damaged cells were found to be sensitive to selective agents (*22*). Other alternative media would include *Listeria* repair broth (LRB), which contains divalent cations (Mn, Mg, and ferric ions), amino acids for protein synthesis, and pyruvate (an oxygen scavenger as injured cells are more sensitive to hydrogen peroxide) to facilitate repair (*5, 6*). Another factor that would increase recovery was use of suboptimal growth temperatures as survival increased as less pressure was put on the repair mechanism (*19*).

CONCLUSIONS

Listeria monocytogenes was documented to enter the VBNC state when subjected to desiccation or under stress conditions. During standard plating of environmental samples that might be contaminated with this organism, questions remained regarding the potential underestimation of populations. From the microscopic fields viewed throughout the two-month period, only one or two VBNC cells were observed among hundreds of other viable and culturable cells. Few or no VBNC cells were observed right after harvesting. At this stage, cells were not starved as they were cultured in tryptic soy broth with yeast extract. Nutrients were available for growth. After being starved in the sand without nutrients, more VBNC cells occurred for about 4 weeks, after which the cells are either viable and culturable or dead. Therefore, *L. monocytogenes* does not generally stay in the VBNC state for >4 weeks during starvation under dry conditions. When plating samples, VBNC cells of *L. monocytogenes* are not biologically significant and did not contribute to underestimation of populations. Many methods and their modifications have been published to recover injured or stressed cells. Incubating the plates anaerobically did not increase recovery. Recovery

with this method was similar to using selective media, which resulted in lower counts. In comparison to the addition of pyruvate as an oxygen scavenger or metabolite substrate, it did not help significantly in the recovery. Use of Columbia agar (CA) instead of TSAYE did not affect the recovery either, even though CA was the base medium for MOX and is much richer in nutrients than TSAYE, thus suggesting better recovery. Adding too much pyruvate (1%) could be detrimental to the cells, hence, the lower recovery obtained compared to 0.1%. Understanding that *L. monocytogenes* does not form VBNC cells under the present stress conditions, confirms that current plating methods are adequate in detecting this pathogen. One of the best and most economical methods to recover the most desiccated stressed cells of *L. monocytogenes* is to use tryptic soy agar with 0.6% yeast extract and aerobic incubation.

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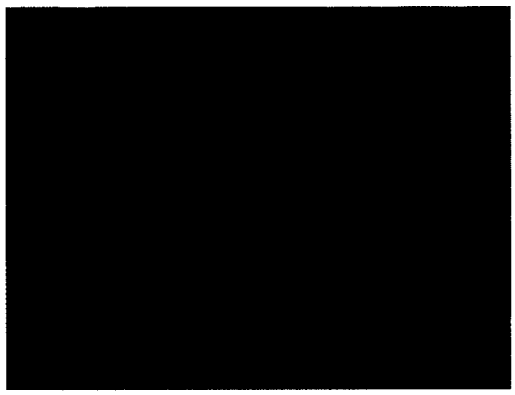
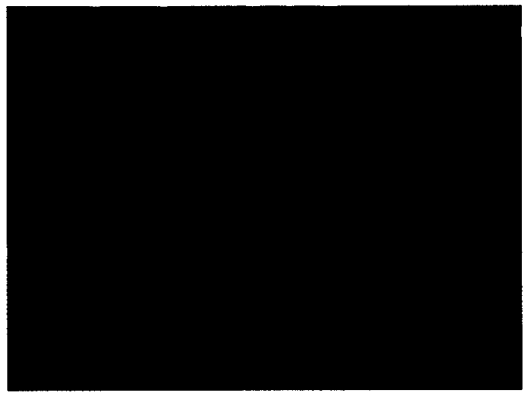
TABLE 2.1. Variations of media used in the recovery of stressed *Listeria monocytogenes* cells

Nonselective Media		Selective Media	
TSAYE	Columbia Agar	MOX	TSAYE + 5% NaCl
Aerobic Anaerobic + 0.1 % pyruvate + 1% pyruvate	Aerobic Anaerobic + 0.1 % pyruvate + 1% pyruvate	Aerobic Anaerobic + 0.1 % pyruvate + 1% pyruvate	Aerobic Anaerobic

(a) Week 0

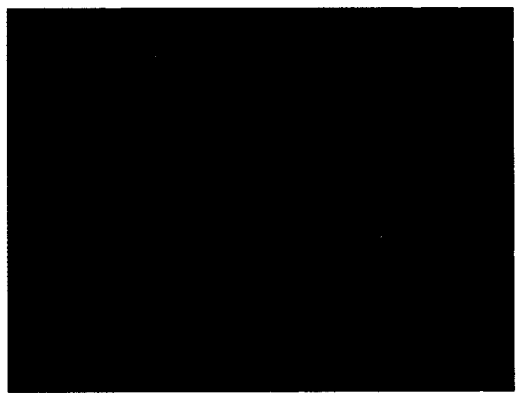


(b) Week 2



(c) Week 4

(d) Week 6



(e) Week 8

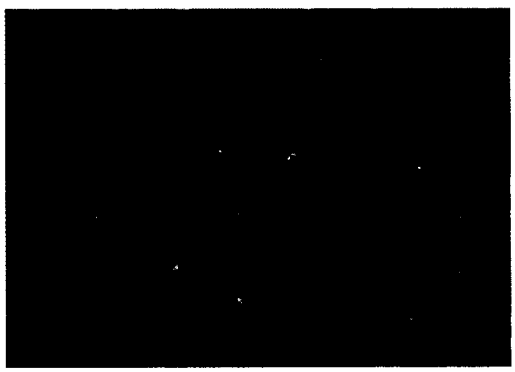
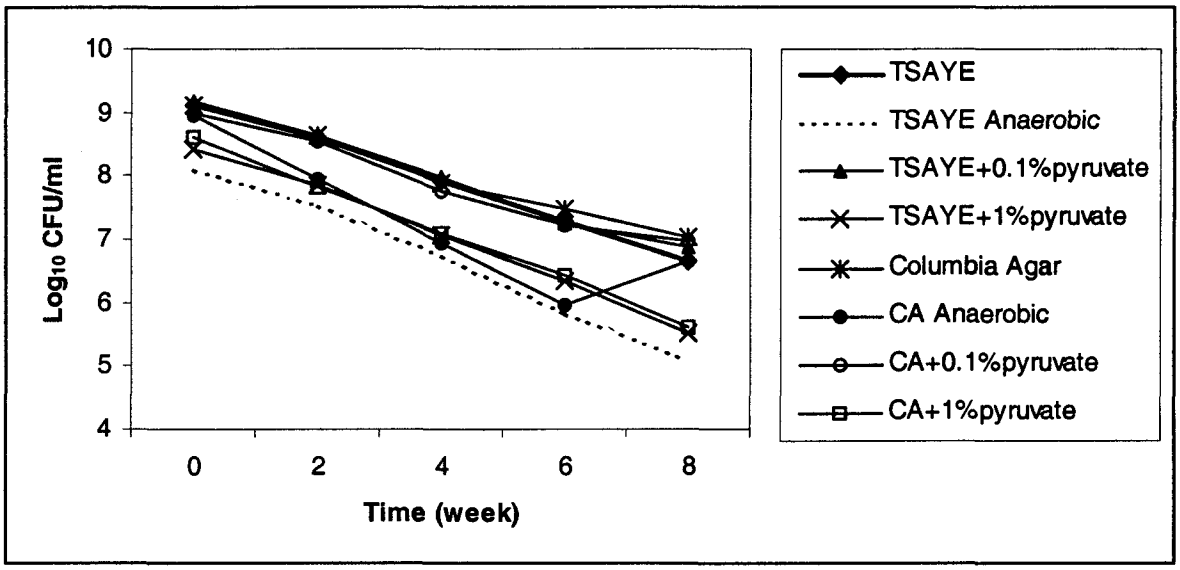


FIGURE 2.1. Viable but nonculturable (VBNC) cells of *Listeria monocytogenes* in the two-month period

(a) Nonselective media



(b) Selective media

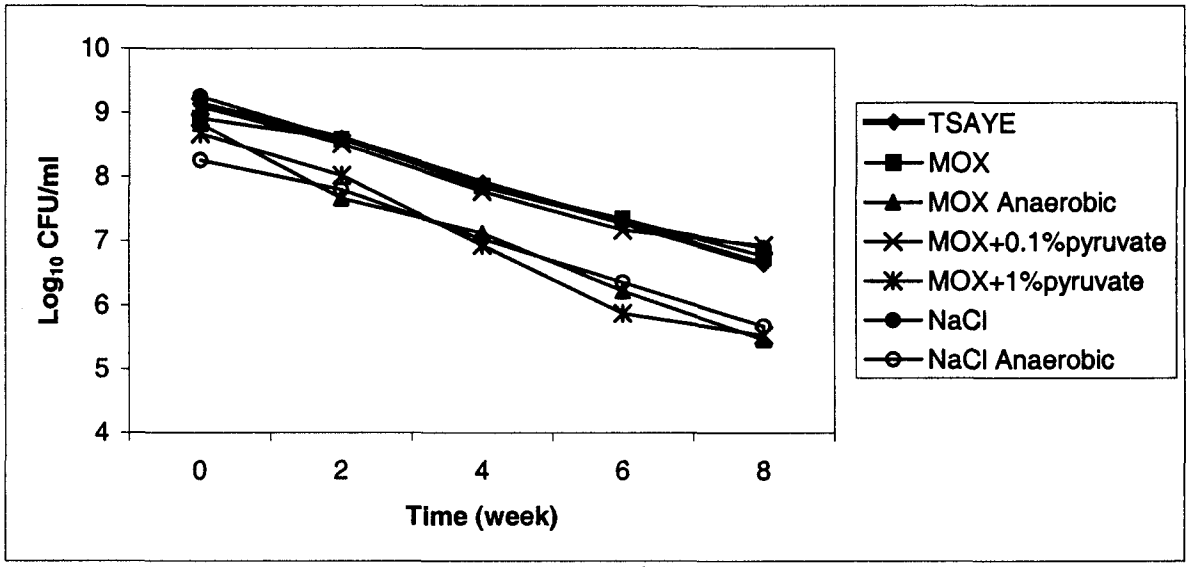


FIGURE 2.2. Comparison of nonselective and selective media in the recovery of stressed induced *L. monocytogenes* with TSAYE as a reference

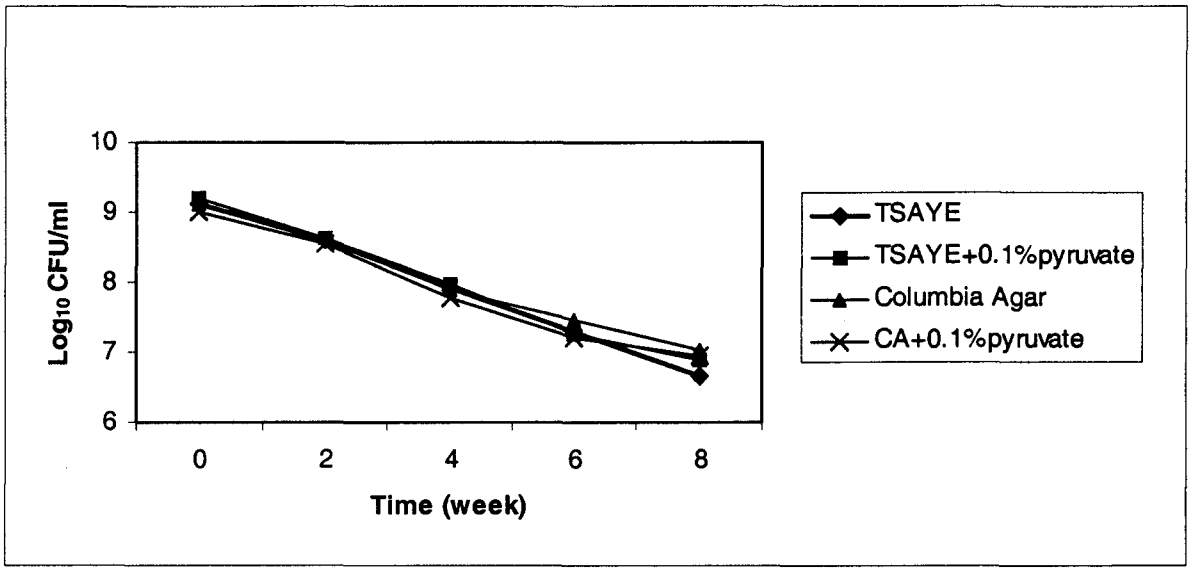


FIGURE 2.3. Comparison of the best four nonselective media in the recovery of stressed induced *L. monocytogenes* with TSAYE as a reference

CHAPTER 3.

ATTACHMENT OF *LISTERIA MONOCYTOGENES* ON READY-TO-EAT (RTE) MEATS

A paper to be submitted for publication in the Journal of Food Protection

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ABSTRACT

Five individual strains of *Listeria monocytogenes* and a mixed cocktail of all five were studied on frankfurters, ham, bologna, and roast beef. Bacterial cell surfaces were characterized by net negative charge and hydrophobicity. Electrostatic interaction chromatography (ESIC) and cationized ferritin (CF) were methods chosen to study net negative charge distribution on the bacterial cell surface. Hydrophobic interaction chromatography (HIC) and contact angle measurement (CAM) were used to examine the cell surface hydrophobicity. The ratio of strongly attached (sessile) *L. monocytogenes* cells compared to total (sessile and planktonic) attached cells on RTE meats was also determined. No differences ($P > 0.05$) were observed in cell surface charge and cell surface hydrophobicity among strains. Approximately 84 to 87% *L. monocytogenes* were found to attach strongly to RTE meats within 5 min. No differences ($P > 0.05$) were found among strains and among meats. Micrographs from scanning electron microscope (SEM) showed no visual difference among the strains but a difference in age of cells (mixed culture) in terms of surface negative charge distribution. More negative sites were observed at day 0

and day 7 and much fewer at day 3, indicating possibly a change in cell surface properties. Since no difference in strains was established, the CAM was carried out using the five-strain mixed culture. Inoculated (10^8 CFU/ml) and noninoculated RTE meats with *L. monocytogenes* were significantly different ($P < 0.05$).

INTRODUCTION

Listeria monocytogenes is a significant foodborne pathogen that causes listeriosis, a disease which has claimed many lives. Ready-to-eat (RTE) processed meats have been associated in many listeriosis outbreaks. From mid 1998 to late 1999 an outbreak occurred with 79 cases in 17 states, and hot dogs and possibly deli meats were incriminated as sources of *L. monocytogenes* (*3, 4*). In 2002, another notable outbreak occurred in northeastern United States due to the consumption of sliced turkey deli meat and resulted in 10 deaths (*5*). The initial phase of contamination of these meat surfaces is presumably bacterial attachment, followed by subsequent survival and growth (*8*). Attachment is a significant step when this pathogenic bacterium adheres to RTE food surfaces, thus, posing a threat if they are consumed without any prior form of heat treatment. Attachment of bacterial cells to surfaces can be affected by cell surface charge, hydrophobicity, hydrophilicity, steric hindrance, roughness, and existence of surface "conditioning layer", structures, flagella, and protein availability (*7, 10, 16, 19*).

Studies on *L. monocytogenes* attachment on processed meats are lacking but are important to understand the fundamental nature of contamination. The United States Department of Agriculture (USDA) requires a 'zero tolerance' for this pathogen on RTE products, which means that processed meats are considered adulterated if any *L.*

monocytogenes is found in these products. The ratio of strongly attached (sessile) *L. monocytogenes* cells compared to total (sessile and planktonic) attached cells on RTE meats was determined. The term S_R value represents the percentage of the total bacterial population physically attached to the tissue surface (*10*). *L. monocytogenes* attachment to adipose tissues was determined to be higher than in lean tissues due in part that fat tissue is hydrophobic thus attached bacteria are not readily washed off (*6*).

Methods selected to determine cell surface charge of *L. monocytogenes* were the electostatic interaction chromatography (ESIC) and use of a dye, cationized ferritin (CF), to study net negative charge distribution under scanning electron microscopy (SEM). Cell surface hydrophobicity, was determined by hydrophobic interaction chromatography (HIC) and contact angle measurement (CAM). This study was undertaken to understand how *L. monocytogenes* attaches to RTE meats and attachment capabilities of this pathogen based on net negative cell surface charge and cell surface hydrophobicity.

MATERIALS AND METHODS

Bacterial cultures and growth conditions. Five individual strains and a mixed cocktail of the five strains of *Listeria monocytogenes* [Scott A (FSRL) culture collection, 1/2a H7764, 4b H7969, 4b H7962, and 4b OB90393] were used in these studies. With the exception of the Scott A strain, all strains were obtained as clinical isolates from the outbreak of 1998 to 1999 (CDC, Atlanta, GA). All strains were grown and maintained on tryptic soy agar slants with 0.6% (wt/vol) yeast extract (TSAYE; Difco Laboratories, Detroit, MI) until needed. Cultures were inoculated into 5 ml tryptic soy broth with 0.6% yeast extract (TSBYE) to revive the cells, followed by a second transfer into 10 ml TSBYE. A five-strain

mixed culture was obtained by mixing 20 µl of the individual strains inoculated into the same tube. These six tubes (five strains and mixed) were incubated 37°C for 24 h. Prior to the studies, the five *Listeria* strains were confirmed positive as *L. monocytogenes* by Gram staining (positive rods) and tested catalase positive and oxidase negative. A rapid ID test, api *Listeria* (bioMérieux Vitek, Inc., Hazelwood, MO) confirmed the cultures to be *Listeria monocytogenes*. These cultures (1 ml) was transferred into a 500 ml of TSBYE in a pyrex bottle and incubated at 37°C with agitation for 14 h. The cells were harvested in the late log phase by centrifugation (3,000 x g for 20 min at 5°C), washed twice with 250 ml Butterfield's phosphate solution (BPS), and were suspended in 50 ml sterile BPS for the chromatography, contact angle measurement, and attachment studies.

Preparation of RTE meats. Four types of commercially available RTE processed meats were selected for use in this study, which include frankfurters, light bologna, chopped ham (water added), deli-style light roast beef (97% fat free), and oven roasted cured beef. The deli-style roast beef was replaced later with the oven-roasted as the former was no longer in production. These meats were sliced in half (excluding frankfurters), repackaged in sets of twelve in Saran vacuum pouches (Racine, WI), and vacuum-sealed using a Multivac machine (Kansas City, MO). The deli-style roast beef was sliced with a Berkel meat-slicer (Laporte, IN). The meats were subsequently irradiated to 7 kGy (Iowa State University Linear Accelerator Facility) using an electron beam source to eliminate most of the naturally occurring microflora and were stored at 4°C until use.

Attachment studies. Procedures for this study were carried out in manners similar to the method described by Dickson and Koohmaraie (1989). Six pieces of each previously irradiated meat type were aseptically transferred to 7.5 in x 11 in Whirl-Pak filter bags

(Nasco, Fort Atkinson, WI). Harvested cells from five individual strains plus a mixed culture of the five-strain were used as inocula. Samples (2 ml) of each harvested bacteria were diluted in 18 ml BPS. Frankfurters were inoculated with the bacteria and BPS mixtures of five individual strains as well as mixed culture for 5 min. Ninety-nine milliliters of BPS were poured into the filter bags and these bags inverted 25 times in 15 s. The bacteria in the buffer were enumerated by plating onto TSAYE in duplicates to determine the number of planktonic cells (loosely) attached. All plating was carried out using the Whitley Automatic Spiral Plater (Don Whitley Scientific Ltd., West Yorkshire, England). The diluent was decanted. Another 99 ml of BPS was added into the same filter bag and homogenized using the Stomacher 400 Lab Blender (Techmar® Company, Cincinnati, OH) on high for 2 min. The bacteria in the diluent were plated onto TSAYE in duplicates using the spiral plater to determine the number of sessile cells (strongly) attached. The TSAYE plates were incubated at 35°C for 48 h. All enumeration of plates was done using the Synbiosis ProtoCOL Colony Counter 3.05 (Sypnotics Ltd., UK). Calculation was S_R values = strongly attached bacteria / (loosely + strongly attached).

Electrostatic Interaction Chromatography (ESIC). This chromatographic method was carried out in manners similar to the method described by Dickson and Siragusa (1994). The ESIC columns were packed with Bio-spin columns for spin chromatography (Bio-Rad, Richmond, CA). Columns were packed with 0.5 g of analytical grade anion exchange Dowex chloride (Fluka Chemical Corp, Milwaukee, WI). The specifications for the Dowex were CL(-)-Form, form 1 x 8, mesh size 100/200, and strongly basic. These columns were washed twice with 1 ml of BPS. Ten microliters of harvested bacteria (5 individual strains and a mixed culture) were suspended into 500 µl BPS and then adsorbed onto the resin. Spin

columns were placed in 50 ml sterile centrifuge tubes (Corning Incorporated, Corning, NY) and centrifuged at 100 x g for 2 min at 5°C. Populations in the initial (10 µl of harvested cells) and the eluted samples were enumerated by plating on TSAYE in duplicates using the spiral plater. These TSAYE plates were incubated at 35°C for 48 h. Values were calculated as the proportion of the $\log_{10}$ population in the resin fraction / $\log_{10}$ population in the eluate fraction. The resin fraction was the initial count of 10 µl of harvested cells prior to inoculation into the columns.

Hydrophobic Interaction Chromatography (HIC). This chromatographic method was carried out in manners similar to the method described by Dickson and Koohmaraie (1989) and Dickson and Siragusa (1994). The same Bio-spin columns (Bio-Rad) were packed with approximately 400 µl of Sepharose® CL-4B (octyl; Fluka Biochemika, Fluka Chemical Corp, Milwaukee, WI) to obtain a gel height of 10 mm. The columns were washed twice with 1 ml of BPS. Ten microliters of harvested cells (five individual strains and a mixed culture) were suspended into 500 µl of BPS and was then adsorbed onto the gel. The spin columns were placed in 50 ml sterile centrifuge tubes (Corning Incorporated) and centrifuged at 100 x g for 2 min at 5°C. Populations in the initial and the eluted samples were enumerated by plating on TSAYE in duplicates using the spiral plater. These TSAYE plates were incubated at 35°C for 48 h and colonies formed were counted. Values were calculated as the proportion of the $\log_{10}$ population in the gel fraction / $\log_{10}$ population in the eluate fraction. The gel fraction was the initial count of 10 µl of harvested cells prior to inoculation into the columns.

Scanning Electron Microscopy (SEM). Following the protocol by Lamed et al. (1987), 500 µl of cells of the mixed *L. monocytogenes* culture were inoculated into 5 ml of 0.9% sodium chloride (NaCl; Fisher Scientific, Springfield, NJ). The suspension was applied to Nuclepore® mixed cellulose ester 47 mm 0.22 µm membrane filter (Whatman Inc., Clifton, NJ). The Nalgene Vacuum Manifold filtering system (Fisher Scientific) was used. The cationized ferritin (CF; 100 mg/ml) used was obtained from Electron Microscopy Sciences (EMS; Fort Washington, PA). This CF, 200 µl (1 mg/ml), was applied to the filter and the interaction between cell surface and CF was allowed to proceed for 10 min. Negative controls were without CF treatment. The filter was washed again with 5 ml of 0.9% NaCl. CF-stained cells mounted to filters and negative control filters were cut using a brass leaf cutter and fixed in 10 ml of 5% glutaraldehyde in 0.9% NaCl at pH 7.2 for 24 h at 4°C. After fixation, the filters were rinsed three times (15 ml each) with buffer. Dehydration was carried out using a graded ethanol (EtOH) series (25%, 50%, 70%, 85% and 95%) with two changes for 10 min each followed by three changes of 100% ethanol, 10 min each. A transition from pure ethanol to pure hexamethyldisilazane (HMDS) was done by a 3:1 (EtOH:HMDS), 1:1, 1:3, to 100% HMDS in 20 min intervals. This was followed by two further changes of pure HMDS for 20 min each. The filters were placed in cracked-lid glass petri dishes under a fume hood and allowed to dry overnight. These filters were then mounted on aluminum stubs with double-sided carbon-coated tape, and sputter coated with gold-palladium for 120 seconds for a coating of approximately 300 Å using a Denton Desk II Sputter Unit (Denton Vacuum, Inc., Terrytown, NJ). Images were captured using a Japan Electron Optics Laboratories (JEOL; Peabody, Mass) JSM-5800LV scanning electron

microscope at 10 kV. Bacteria were taken at 0, 3, and 7 days post harvesting of cells and stored in 10 ml of BPS at 4°C to determine the effects of age differences.

Contact Angle Measurement (CAM). The sessile drop technique was carried out using a method similar to that described by James and Brown (1986). Since no difference ($P > 0.05$) was found among the attachment of the five individual strains, CAM was carried out using a mixed culture of all five strains. Harvested cells of *L. monocytogenes* were inoculated (100 µl) onto sterile Nuclepore® mixed cellulose ester 47 mm 0.22 µm membrane filter (Whatman Inc., Clifton, NJ) and onto the surface of the selected processed RTE meat (frankfurters, sliced bologna, sliced chopped ham, and sliced oven roasted cured roast beef). The sliced meats were cut into 5 x 5 cm^2 while the frankfurters into 2.5 cm in length. These meats were surface inoculated and evenly distributed with 100 µl (10^8 CFU/ml) of harvested *L. monocytogenes* cells. Inoculated filters and meats were placed in petri plates, placed in the cooler at 4°C for 24 h to slightly dry out. The controls were uninoculated meat samples and membrane filter. All the samples were removed from the cooler and placed on the workbench with the petri plate caps removed. Filter and meat samples were exposed to the air for 4 h. Twenty microliters of sterile distilled water were dropped onto each of the filter and meat type surfaces. At the moment of contact (within 1 s), the drop was photographed with macro lens (*7, 11*) using Elite chrome 200 slide film (Eastman Kodak Company, Rochester, NY). Photographs of contact angles were taken every 30 min for 4 h. The developed photographic slides were projected and contact angles measured.

RESULTS AND DISCUSSION

Attachment to RTE meat surfaces. Table 3.1 indicates the S_R value, which represents the ratio of strongly attached (sessile) cells to total cells (sessile and planktonic) attached to each ready-to-eat (RTE) meat surface by each strain or mixed culture of *Listeria monocytogenes*. Results showed no significant difference ($P > 0.05$) in the attachment of the five different strains and the mixed culture used in the experiment. No significant differences ($P > 0.05$) were observed in the attachment of *L. monocytogenes* among the different selected RTE meat types, frankfurters, slice bologna, sliced chopped ham, and sliced deli-style roast beef. The RTE meats chosen represent different raw materials and processing types, for instance, frankfurters and bologna are cooked comminuted products, ham is cured, and roast beef is made from intact muscle. Based on these results, attachment of *L. monocytogenes* was not dependent on strain or type of RTE meat. A relative high percentage of strong attachment was found on RTE meats. Approximately 84 to 87% of *L. monocytogenes* were observed to attach strongly (sessile cells) to the selected RTE meats within 5 min. These sessile cells would be harder to remove physically, as they would bind irreversibly to surfaces and would not be detached by normal processing conditions.

Electrostatic Interaction Chromatography (ESIC). The relative net negative surface charges expressed as *r/e* values of five individual *L. monocytogenes* strains and a mixed culture of the five (Table 3.2). No significant difference ($P > 0.05$) was observed among the individual strains and the mixed culture. An increase in *r/e* value indicates an increase in the amount of negative surface charges of the bacteria assayed (*18*). Even though the strains studied had similar relative surface charges, attraction and repulsion of positive and negative charges exist between bacteria and surfaces which would affect attachment by

other bacterial species and different surface substrata (*10*). An increase in attachment had been associated with a relative low surface energy and also an increase in cell surface charge, although there are mixed reports available in terms of the relationship between surface negative charge and attachment capabilities (*10, 17, 20*).

A net negative charge (electron donor) exists on the cell wall of bacterial cells, which can be characterized by electrostatic interaction chromatography (ESIC), that is, the bacterial affinity for the ion exchanges (*2, 10, 18*). This net negative charge plays an important role in bacterial attachment to lean meat tissues, in that greater net negative charge of cell surface has been positively correlated to *L. monocytogenes* attachment to beef tissue surfaces (*9, 10*). Electrokinetic potential increases possibly with decreasing hydrophobicity (*20*).

Hydrophobic Interaction Chromatography (HIC). The relative surface hydrophobicities expressed as *g/e* values of *L. monocytogenes* (Table 3.2). No significant difference ($P > 0.05$) was observed for the five individual strains and the mixed culture of *L. monocytogenes*. An increase in *g/e* value indicates an increase in the degree of bacterial surface hydrophobicity, which further indicates more extensive attachment (*17*). Bacterial adhesion was more extensive to hydrophobic surfaces of relatively low surface energy (*10, 17, 20*).

Cell surface hydrophobicity is affected by temperature, pH, time, and bacterial growth and concentration (*15, 20*). *L. monocytogenes* was found to have stronger attachment on hydrophobic compared to hydrophilic surfaces and hydrophilic uncharged surfaces showed higher resistance to cell attachment (*7*). Growth temperature and pH affect cell surface hydrophobicity, however, the differences in hydrophobicity could not be correlated to differences in rates of attachment (*19*). Growth conditions influence cell surface

characteristics for adhesion and hydrophobicity of cells increases with higher growth rates (*20*).

Scanning Electron Microscopy (SEM). The net negative surface charge distributions of *L. monocytogenes* are illustrated in Figures 3.1 and 3.2. The SEM micrographs provide graphic representation of *Listeria monocytogenes* attachment to surfaces based on the net negative surface charge distribution. Cationized ferritin (CF) has been used to better visualize exocellular structures (protuberances) by binding to anionic compounds found on microbial cell surface showing potential sites for attachment (*1, 14*). From observing the micrographs, no strain difference in charge distribution was noted (Fig. 3.1). Figure 3.1 also showed the difference between a control of *L. monocytogenes* without CF treatment and the other strains or mixed culture treated with CF indicated by the protuberances. These protuberances were indicative of the cell surface negative charge sites, which were most visually pronounced with the mixed culture. Figures 3.1 and 3.2 indicated attachment showing extracellular fibrils (perhaps condensed exopolysaccharide) between cells (*13*).

The age of the culture did affect charge distribution. The older the culture, that is, cells harvested and stored for two weeks compared to freshly harvested one, had less binding of ferritin. Figure 3.2 illustrates the use of a mixed culture to study surface charge differences caused by age of the cultures. Visual observations of these SEM micrographs consistently indicated a slight decrease in net negative surface charge distribution at day 3 compared to the ones for day 0 and day 7, possibly due to some changes in the cell surface. X-ray spectra with peaks (Kα-Fe) indicating binding of ferritin for days 0, 3, and 7 compared to control (without CF) are shown in Figure 3.3.

Contact Angle Measurement (CAM). There was a significant difference ($P < 0.05$) between inoculated (10^8 CFU/ml) and non-inoculated RTE meats (Table 3.3). Inoculation onto filter paper (control for surfaces) and roast beef showed a decrease in hydrophobicity, an increase for frankfurters, and unchanged for ham and bologna compared to non-inoculated ones. Frankfurters had higher hydrophobicity compared to the other RTE meats. Frankfurters are chopped, blended, and stuffed, hence the surface is more uniform. After cooking and peeling, coagulated protein is formed on the surface increasing the hydrophobicity. In addition, with the higher fat content, these factors would increase hydrophobicity.

CONCLUSIONS

Understanding how *Listeria monocytogenes* attaches to surfaces, especially food that is not heated prior to consumption, is important in giving insights into the fundamental nature of contamination. Although the strains used in these studies were of different serotypes, they showed no significant difference ($P > 0.05$) in terms of attachment to meat surfaces based on the S_R values, cell surface negative charge, and cell surface hydrophobicity. Some previous experiments have indicated differences in strains in their attachment characteristics to different surfaces, food or equipment. One theory was that certain serotypes attach to meats better than others. However, this research indicated otherwise. The time at which the organism comes in contact with the surface until irreversible attachment occurs is important, and strong attachment to the meats by the cells occurred within 5 min. The initial numbers of contaminants are directly proportional to the number of organisms attached. Other factors that affect attachment include bacterial species, type of surfaces (organic and inorganic),

formation of flagella or EPS layer, growth phase of the organism, and age of the culture. This study showed a slight difference in the charge distribution visually observed from the cationized ferritin-stained SEM micrographs. A change in surface charge occurred three days after the cells were harvested which increased again by day 7. Perhaps young cultures need some time to adapt to the storage conditions which were in buffer solution and at cold temperatures. Ssome stresses (nutrient deficiencies and cold) were being exerted on the cells which need to be overcome. With increase charged sites and EPS, attachment could be facilitated with older cultures. Therefore, cells stored in cold, nutrient deprived environments may have facilitated attachment. Contact angle measurements indicated surface hydrophobicities of different processed meat surfaces. Frankfurters were definitely more hydrophobic than the sliced meats. Roast beef proved to be the most difficult to determine contact angles. Since these are intact muscles, droplets of water, at most times, just went right through without forming any angles. Inoculation of meat surfaces with *L. monocytogenes* changed the surface hyrdophobicity. Bacterial cells have a net negative surface charge and tend to attach to surfaces with high hydrophobicity. *L. monocytogenes* was shown to be hydrophilic from physiochemical characterization and CAM (*17*). These properties cannot be changed. However, manipulation of meat or food contact surfaces to make them less hydrophobic such as organic acid sprays or dips, attachment of *L. monocytogenes* can be decreased hence enhancing safety of foods, especially RTE meats that do not require preheating before consumption. Taken together these experiments indicate that *L. monocytogenes* in the environment and once in contact with RTE processed meats can attach strongly and rapidly.

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TABLE 3.1. S_R values of five individual strains and a mixed culture of *Listeria monocytogenes* attachment on selected RTE meats[a]

Strain	Frankfurter	Bologna	Ham	Roast Beef
Scott A	0.86 ± 0.004	0.86 ± 0.022	0.84 ± 0.005	0.86 ± 0.002
1/2a H7764	0.87 ± 0.001	0.85 ± 0.006	0.85 ± 0.008	0.87 ± 0.006
4b H7969	0.86 ± 0.022	0.85 ± 0.012	0.84 ± 0.004	0.85 ± 0.005
4b H7962	0.85 ± 0.007	0.85 ± 0.008	0.85 ± 0.002	0.85 ± 0.006
4b OB90393	0.85 ± 0.004	0.84 ± 0.007	0.86 ± 0.014	0.85 ± 0.004
Mixed culture	0.86 ± 0.009	0.85 ± 0.009	0.85 ± 0.007	0.86 ± 0.005

[a] Mean and standard error of the mean (SEM) of each RTE meat type for each strain from three separate studies. No significant differences determined using ANOVA ($\alpha = 0.05$)

TABLE 3.2. Cell surface negative charge (ESIC) and cell surface hydrophobicity (HIC) values for five individual strains and a mixed culture of *L. monocytogenes*[a]

Strain	ESIC	HIC
Scott A	0.38 ± 0.061	0.21 ± 0.027
1/2a H7764	0.44 ± 0.078	0.26 ± 0.008
4b H7969	0.43 ± 0.053	0.25 ± 0.015
4b H7962	0.37 ± 0.026	0.24 ± 0.010
4b OB90393	0.41 ± 0.055	0.23 ± 0.008
Mixed culture	0.40 ± 0.041	0.23 ± 0.007

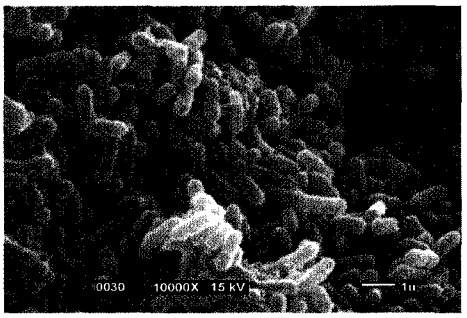
[a] Mean and standard error of the mean (SEM) of each chromatographic method for each strain from three separate studies. No significant differences determined using ANOVA ($\alpha = 0.05$)

TABLE 3.3. Contact angle measurements (CAM) for membrane filter and selected RTE meats inoculated and not inoculated with a mixed culture of *L. monocytogenes*[a]

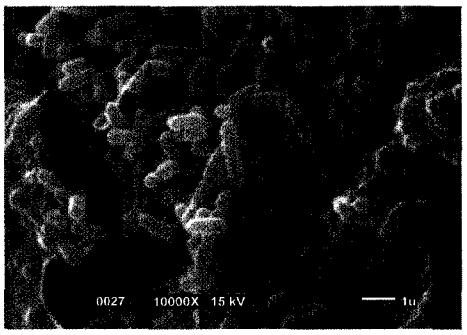
Surface substrata	Noninoculated (°)	Inoculated (°)
Membrane Filter	54 ± 2	25 ± 1
Frankfurter	29 ± 2	34 ± 5
Bologna	20 ± 1	19 ± 2
Ham	19 ± 1	17 ± 2
Roast Beef	20 ± 1	13 ± 2

[a] Mean and standard error of the mean (SEM) of noninoculated and inoculated *L. monocytogenes* on different surface substrata from three separate studies.

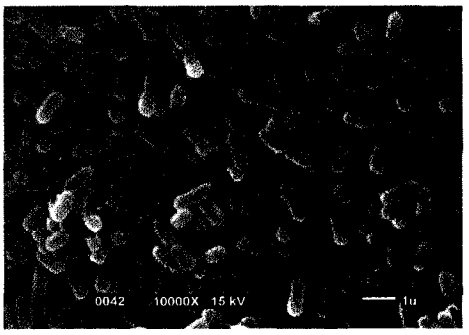
(a) Control, without cationized ferritin (CF)



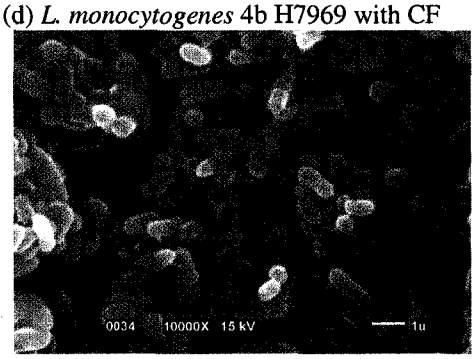
(b) *L. monocytogenes* Scott A with CF



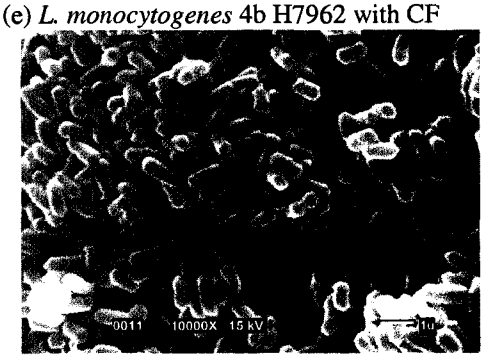
(c) *L. monocytogenes* 1/2a H7764 with CF



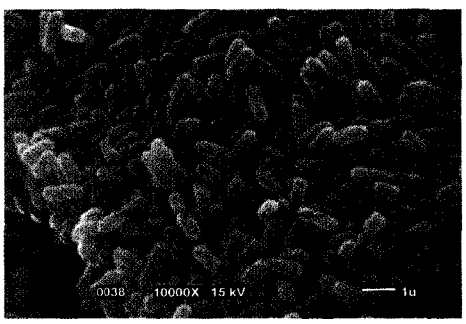
(d) *L. monocytogenes* 4b H7969 with CF



(e) *L. monocytogenes* 4b H7962 with CF



(f) *L. monocytogenes* 4b OB90393 with CF



(g) Mixed culture of all five strains with CF

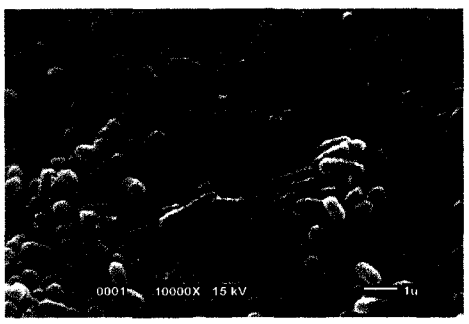
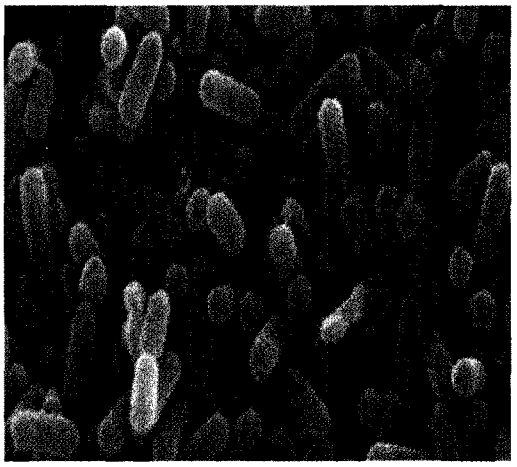
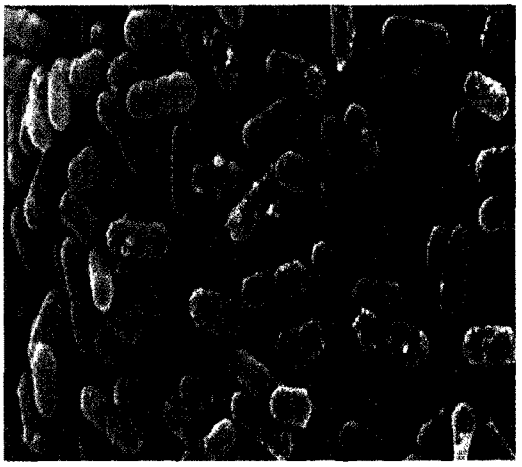


FIGURE 3.1. Cell surface negative charge distribution and extracellular fibrils on five individual strains and a mixed culture of *Listeria monocytogenes*

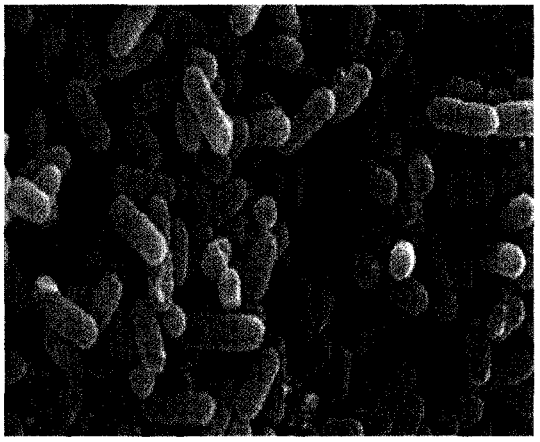
(a) Control, without cationized ferritin



(b) Treated with cationized ferritin at day 0



(c) Treated with cationized ferritin at day 3



(d) Treated with cationized ferritin at day 7

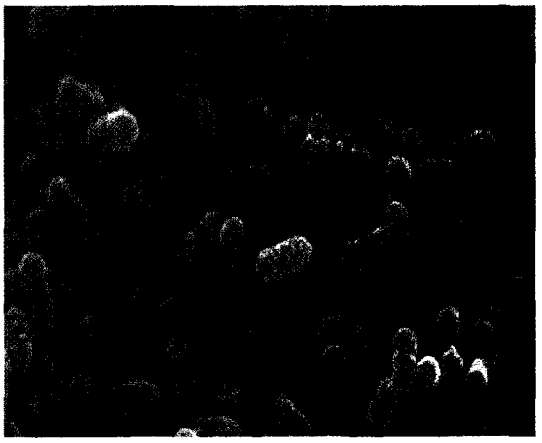
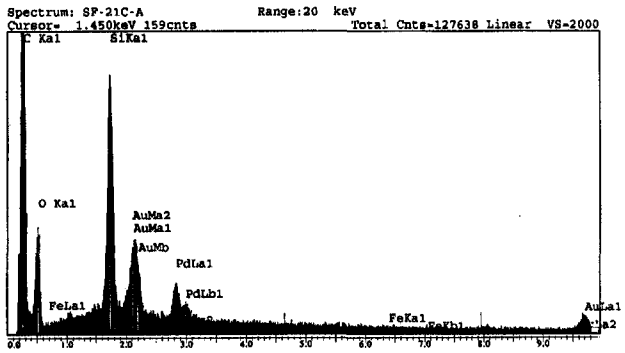
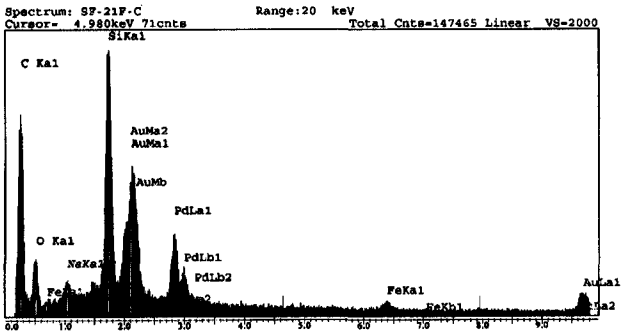


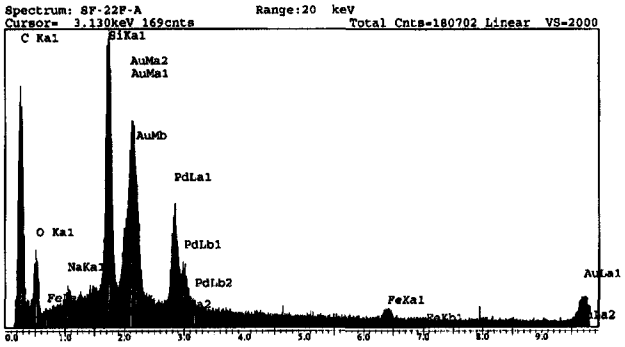
FIGURE 3.2. Age difference of cell surface negative charge distribution of *L. monocytogenes*



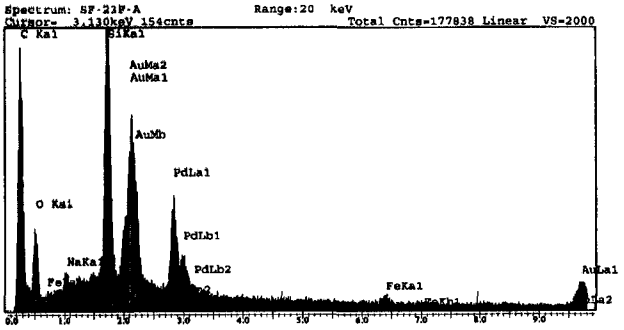
(a) Control



(b) Day 0



(c) Day 3



(d) Day 7

FIGURE 3.3. X-ray spectra illustrating binding of cationized ferritin (Kα-Fe) to *L. monocytogenes*

CHAPTER 4.

REDUCTION AND SURVIVAL OF *LISTERIA MONOCYTOGENES* IN READY-TO-EAT (RTE) MEATS AFTER IRRADIATION

A paper to be submitted for publication in the Journal of Food Protection

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ABSTRACT

A five-strain *Listeria monocytogenes* culture was inoculated onto six different types of ready-to-eat (RTE) meats (frankfurters, ham, roast beef, bologna, smoked turkey with lactate, and smoked turkey without lactate). The meats were vacuum-packed and stored at 4°C for 24 h prior to irradiation. Populations of *L. monocytogenes* were recovered by surface plating on nonselective and selective media. The margins of safety studied include 3-$\log_{10}$ (3D) and 5-$\log_{10}$ (5D) reduction of pathogenic bacteria to achieve an optimal level of reduction while retaining organoleptic qualities of the meats. The doses stated are based on doses targeted for irradiation. A 3-$\log_{10}$ reduction of *L. monocytogenes* was obtained at 1.5 kGy when nonselective plating medium was used. The dosages for 3-$\log_{10}$ reduction were 1.5 kGy for bologna, roast beef, and both types of turkey, and 2.0 kGy for frankfurters and ham based on use of selective medium. The D_{10} values ranged from 0.42 to 0.44 kGy. A 5-$\log_{10}$ reduction of *L. monocytogenes* was obtained at 2.5 kGy with nonselective medium. Using selective medium, the dosages were 2.5 kGy for bologna, roast beef, and both types of turkey, and 3.0 kGy for frankfurters and ham. Survival of *L. monocytogenes* in the same

RTE meat types after irradiation was also studied. Meats were inoculated with 5 $\log_{10}$ of *L. monocytogenes*/g and irradiated at doses of 2.0 and 4.0 kGy. Recovery of the surviving organisms was observed during storage at temperatures of 4°C and 10°C for 12 weeks. Preliminary results showed no growth in meats irradiated at 4.0 kGy. Survivors were observed for irradiated meats at 2.0 kGy stored at 10°C after the second week. No growth was observed in samples irradiated at 2.0 kGy stored at 4°C until the fifth week.

INTRODUCTION

Listeria monocytogenes is considered a foodborne pathogen of great public health significance (*3, 5, 16*). *L. monocytogenes* has been incriminated in numerous outbreaks associated with ready-to-eat (RTE) meats. A significant outbreak occurred with frankfurters in 1998 and 1999, which resulted in 21 fatalities and approximately 100 reported cases of listeriosis (*1*). In 2002, another notable outbreak occurred in northeastern United States resulting in 10 deaths due to consuming sliced turkey deli meat (*2, 4*). The United States Department of Agriculture (USDA) requires a "zero tolerance" for *L. monocytogenes* in RTE processed meats and this bacterium is considered to be a post-processing contaminant (*7, 9, 17*). *Listeria* is well known for its resistance to several environmental stresses, such as refrigeration or freezing temperatures, heat, low pH, desiccation, and high salt and nitrite concentrations (*12, 18*). Of particular interest is its ability to grow at refrigeration temperatures and in environments with increased salt concentrations, which depict conditions commonly associated with RTE meats (*14*). RTE meats by definition, are often consumed without a final thermal process by the consumers (*8*).

Many technologies have been developed to curb this problem of post-processing contamination. One such method is use of low dose irradiation (*6, 11*). A study group from the World Health Organization (WHO) concluded that food irradiated to any dose 'appropriate to achieve the intended technological objective' is both safe to consume and nutritionally adequate. The microbiological objectives are to improve safety, reduce initial population, and extend shelf life. Decimal reduction values, or D_{10} values, estimate the radiation dose (in kGy) needed to reduce bacterial numbers by 90% or a one-$\log_{10}$ reduction (*13, 14*). *L. monocytogenes* is fairly susceptible to irradiation. Various D_{10} values have been reported because they are dependent on strain, substrate, irradiation type, and plating medium (*12, 15*). However, only a few published investigations have described the effects of irradiation on processed meats. High dose irradiation has a negative effect on the organoleptic qualities of the meat (*10, 14*). Therefore, an optimal dose is required for margin of safety while preserving the quality of meat. Low dose gamma irradiation (<5 kGy) was speculated to eliminate pathogens while maintaining sensory qualities of frankfurters (*14*). In using the lower doses, there may be a chance for *L. monocytogenes* to survive and grow. In order to look at this issue further, an experiment was conducted to study the survival and growth of this pathogen on selected RTE meats stored at refrigeration temperatures.

This investigation was conducted (i) to determine the irradiation doses required for a 3- and 5-$\log_{10}$ reduction of *L. monocytogenes* on selected RTE meats which are frankfurters, sliced bologna, sliced chopped ham, sliced roast beef, sliced smoked turkey with lactate, and sliced smoked turkey without lactate; (ii) to determine the D_{10} values for *L. monocytogenes* using an electron beam source on the same RTE processed meats indicated; and (iii) to study the post irradiation survival and growth of *L. monocytogenes* in RTE meats; frankfurters,

bologna, turkey ham, and roast beef. Two varieties of smoked turkey were used, with or without lactate, which is known to be an anti-listerial processing aid. A comparison was done to observe if a difference in irradiation dose is required to reduce the number of cells.

MATERIALS AND METHODS

Culture conditions. A five-strain culture of *Listeria monocytogenes* [Scott A (FSRL culture collection), 1/2a H7764, 4b H7969, 4b H7962, and OB90393] was used in this experiment. Each strain was grown individually in 10 ml tryptic soy broth containing 0.6% yeast extract (TSBYE; Difco Laboratories, Detroit, MI) for 24 h at 37°C. The strains were then combined by adding 1 ml of each to 500 ml of TSBYE and incubated at 37°C (100 rpm) with agitation for 14 h. These cells were harvested, washed twice, and suspended into 50 ml of Butterfield's phosphate solution (BPS), resulting in an inoculation cocktail of approximately 10^{10} cells/ml. The experiment was independently replicated three times.

Preparation of ready-to-eat meats. Background flora of *L. monocytogenes* was tested to be negligible (data not shown) on the selected commercially available pieces/slices of meats (frankfurters, chopped ham, bologna, turkey ham, roast beef, smoked turkey with lactate, and smoked turkey without lactate). In order to determine the D_{10} values, these meats were individually transferred to sterile filtered stomacher bags and inoculated with 1 ml of the *L. monocytogenes* cocktail. These bags were vacuum-packed and stored at 4°C for 24 h prior to irradiation at the appropriate doses. For the survival and growth study, 25 g of the meat samples were weighed into sterile stomacher bags and then surface-inoculated with 1 ml of a previously diluted solution containing approximately 10^5 CFU/g of *L.*

monocytogenes. Samples were then vacuum-packed and stored at 4°C for 24 h prior to irradiation. Non-irradiated samples were used as controls.

Irradiation. Irradiation was carried out at the Iowa State University Linear Accelerator Facility (LAF) using an electron beam source. Doses selected for the irradiation reduction and the D_{10}-value experiments ranged from 0 to 4 kGy with 0.5 increments. For the study of the survival and growth of *L. monocytogenes* after irradiation, the doses selected were 2 and 4 kGy and these meats were stored at 4°C and 10°C for 12 weeks.

Sampling and enumeration. For the irradiation reduction and D_{10}-value experiments, 20 ml of BPS was added and meat samples were homogenized in the Stomacher 400 Lab Blender (Techmar® Company, Cincinnati, OH) for 2 min. Appropriate dilutions were made and surface plated using a spiral plater onto tryptic soy agar with 0.6% yeast extract (TSAYE) and modified Oxford agar (MOX) in duplicates. Plates were incubated at 37°C for 48 h to enumerate total plate counts. For the survival study, inoculated and irradiated meat samples were stored at 4°C and 10°C and sampled once a week for 12 weeks. For the different bags with meat sampled, 225 ml of 0.1% sterile peptone water were added to the 25 g meat sample and homogenized for 2 min in the stomacher. Appropriate dilutions were made and surface plated in duplicates onto TSAYE and MOX. Plates were incubated at 37°C for 48 h and then enumerated using the Synoptics Ltd. (UK) ProtoCOL (model 60000) automated plate counter.

Calculation of D_{10} values. D_{10} values were determined by plotting the irradiation dose (kGy) against the average $\log_{10}$ CFU/g from each dose in the duplicate samples of three independent trials. Linear regression curves were generated using a statistical software,

JMP™ (SAS Institute Inc., Cary, NC), where the D_{10} values were determined as the absolute value of the reciprocal of the slope of the curve.

RESULTS AND DISCUSSION

Irradiation reduction. Irradiation of processed meats is pending for approval. At doses around 2 kGy, the irradiation process caused very distinctive changes in the aroma, flavor, and texture of the meats that make them very unappealing for consumption. Moreover, *L. monocytogenes* post-processing contamination from the environment would not achieve high counts that would require high dose irradiation for safety. Approximately 3- or 5-$\log_{10}$ reduction using lower doses may be sufficient to achieve a margin of safety for processed meats. Tables 4.1 and 4.2 illustrate estimated doses required for specific reductions based on nonselective medium (TSAYE) and on a selective medium (MOX). A 3-$\log_{10}$ reduction of *L. monocytogenes* on the nonselective medium was obtained at 1.5 kGy (actual doses: 1.89 ± 0.12 kGy for frankfurters; 1.60 ± 0.09 kGy for sliced meats). On selective medium, the doses were 1.5 kGy (actual dose: 1.60 ± 0.09 kGy) for bologna, roast beef, and both types of smoked turkey, and 2.0 kGy for frankfurters (actual dose: 2.43 ± 0.13 kGy) and ham (actual dose: 2.07 ± 0.14 kGy). A 5-$\log_{10}$ reduction of *L. monocytogenes* on the nonselective medium was obtained at 2.5 kGy (actual doses: 3.20 ± 0.16 kGy for frankfurters; 2.70 ± 0.24 kGy for sliced meats). On selective medium, the doses were 2.5 kGy (actual dose: 2.70 ± 0.24 kGy) for bologna, roast beef, and both types of smoked turkey, and 3.0 kGy for frankfurters (actual dose: 3.96 ± 0.09 kGy) and ham (actual dose: 3.30 ± 0.25 kGy). The 0.5 kGy difference for frankfurters from the selective media could be due to the thickness (ca. 1.5 cm), a higher irradiation dose is generally required for effective

penetration of electrons compared to the sliced meats. As for ham, the meat and fat were finely chopped and not blended well, which might have affected the higher dose required. When this higher dose was combined with the selective pressures of selective media, higher doses were observed for both the 3- and 5-$\log_{10}$ reduction of *L. monocytogenes*. The irradiation doses found from this experiment were below the one reported (3.55 kGy) for a 5-$\log_{10}$ reduction of *L. monocytogenes* from gamma-irradiated frankfurters (*14*).

D_{10} value. Using the irradiation reduction procedure, the D_{10} values for the meats could be obtained by plotting the values on a straight line and the D_{10} values calculated from the slope. The D_{10} values for frankfurters, bologna, ham, and roast beef ranged from 0.42 to 0.44 kGy with an average of 0.44 kGy when using the nonselective medium TSAYE (Fig. 4.1). These doses were lower than previously reported by Sommers and Thayer (*14*), which ranged from 0.49 to 0.71 kGy with an average of 0.61 kGy based on surface-inoculated frankfurters, vacuum-packed, and gamma irradiated.

Survival and growth. Survival and growth of *L. monocytogenes* in the selected RTE meats after irradiation at doses of 2 and 4 kGy were studied during storage at temperatures of 4°C and 10°C for 12 weeks. Figure 4.2 showed the projected growth of *L. monocytogenes* on RTE processed meats stored at 10°C using the USDA-ARS Pathogen Modeling Program (PMP). No survivors were observed in samples irradiated at 4 kGy. A dose of 2 kGy was successful in reducing the numbers of *L. monocytogenes* and storage at 4°C proved to be effective in suppressing the growth of the organism for about 5 weeks after irradiation. As expected, storage at 10°C allowed the growth of higher numbers of *L. monocytogenes* in all the selected RTE meats. Figures 4.3 to 4.5 illustrated the survival and growth of *L. monocytogenes* on the selected RTE meats stored at 4°C and 10°C.

Prolonged lag phases after the RTE meats were irradiated are shown in Figures 4.3 to 4.5. An extended lag phase was observed with selected processed meats irradiated at 2 kGy and especially for the ones stored at 4°C. When bologna and turkey ham were irradiated at 2 kGy and stored at 4°C, the lag phase increased 4 weeks and 2 weeks, respectively. This extended shelf life is important meaning that the irradiated meats can be stored for that much longer time before spoilage. Increase in lag phase corresponds with an increase in irradiation dose but less pronounced with temperature increase. This is due to high irradiation dose injury of the cells and recovery from damage takes longer at lower temperatures (*12*). Patterson *et al.* (*12*) conducted a similar study using gamma-irradiated cooked poultry meat and found the lag phase was extended to 18 d for meat irradiated at 2.5 kGy compared to 1 d for non-irradiated when stored at 6°C. Roast beef was found to be more sensitive to radiation earlier in this investigation (data not shown). Product formulation and processing may play a role in the effect of irradiation on foodborne pathogens (*14*). In this experiment, *L. monocytogenes* exhibited minimal or no growth when examined in roast beef samples. Therefore, only data for the survivors growing on bologna, frankfurters, and turkey ham are presented (Figs. 4.3 to 4.5).

No apparent difference in generation time was observed. No apparent difference was found in the maximum population densities (although 2 kGy samples consistently had lower MPD's; Fig. 4.2).

CONCLUSIONS

Obtaining an optimal dose of irradiation to achieve a margin of safety of processed meats is essential. Having this information helps meat processors obtain a safe product and

at the same time maintain product quality. Estimated dose for a 3-$\log_{10}$ reduction of *L. monocytogenes* on selected RTE meats is 1.5 kGy and a 5-$\log_{10}$ reduction is 2.5 kGy (data from nonselective medium). No apparent difference was observed between the irradiation D_{10} values in the smoked turkey with lactate and without lactate. The counts obtained were comparable which indicated the same irradiation dose is required for a 3- and a 5-$\log_{10}$ reduction. The average D_{10} value for *L. monocytogenes* on the selected meats, frankfurters, bologna, ham (pork and turkey), roast beef, and both types of smoked turkey, is 0.44 kGy. These estimates provide information as to the approximate irradiation dose required for securing the safety of processed meats. Depending on the initial inoculum or contamination level, the irradiation doses can be adjusted to be sufficient to eliminate this pathogen while preserving the qualities of the meats as well. As for the survival and growth of *L. monocytogenes* on selected processed meats, no recoverable bacterium was found at 4 kGy stored at either temperature (4°C or 10°C). The results from this study provide RTE meat processors a better idea on the use and effects of irradiation as part of the unit operations in securing the safety of such meats that do not have a final heat kill step before consumption.

ACKNOWLEDGMENTS

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TABLE 4.1. *L. monocytogenes* (log_{10} CFU/ml) recovered from meats irradiated at specific doses providing estimated doses required for specific reductions based on nonselective medium, TSAYE

Dose (kGy)	Franks	Bologna	Ham	Roast beef	Smoked turkey with lactate	Smoked turkey without lactate
0	**9.60**	**9.63**	**9.48**	**9.39**	**9.86**	**9.89**
0.5	8.25	8.27	8.06	8.07	8.67	8.12
1	7.59	7.66	7.39	7.34	8.02	7.22
1.5	**6.51**	**6.76**	**6.67**	**6.37**	**6.90**	**6.10**
2	5.41	5.81	5.70	5.56	5.64	5.03
2.5	**4.43**	**4.44**	**4.48**	**4.41**	**4.43**	**4.93**
3	3.83	3.39	3.80	3.12	3.93	3.60
3.5	1.07	1.97	0	0	3.77	0
4	0	0	0	0	0	0

TABLE 4.2. *L. monocytogenes* (log_{10} CFU/ml) recovered from meats irradiated at specific doses providing estimated doses required for specific reductions based on selective medium, MOX

Dose (kGy)	Franks	Bologna	Ham	Roast beef	Smoked turkey with lactate	Smoked turkey without lactate
0	**8.84**	**9.31**	**8.49**	**8.99**	**9.86**	**9.90**
0.5	7.82	7.47	7.40	7.52	8.54	8.23
1	7.15	6.94	6.85	6.38	7.91	7.12
1.5	6.02	**6.33**	5.91	**5.36**	**6.78**	**6.01**
2	**5.57**	5.55	**5.54**	4.96	5.65	5.01
2.5	4.62	**4.28**	4.21	**4.01**	**4.40**	**4.74**
3	**3.76**	3.71	**3.40**	2.70	3.79	3.61
3.5	0	0.77	0	1.63	3.41	0
4	0	0	0	0	0	0

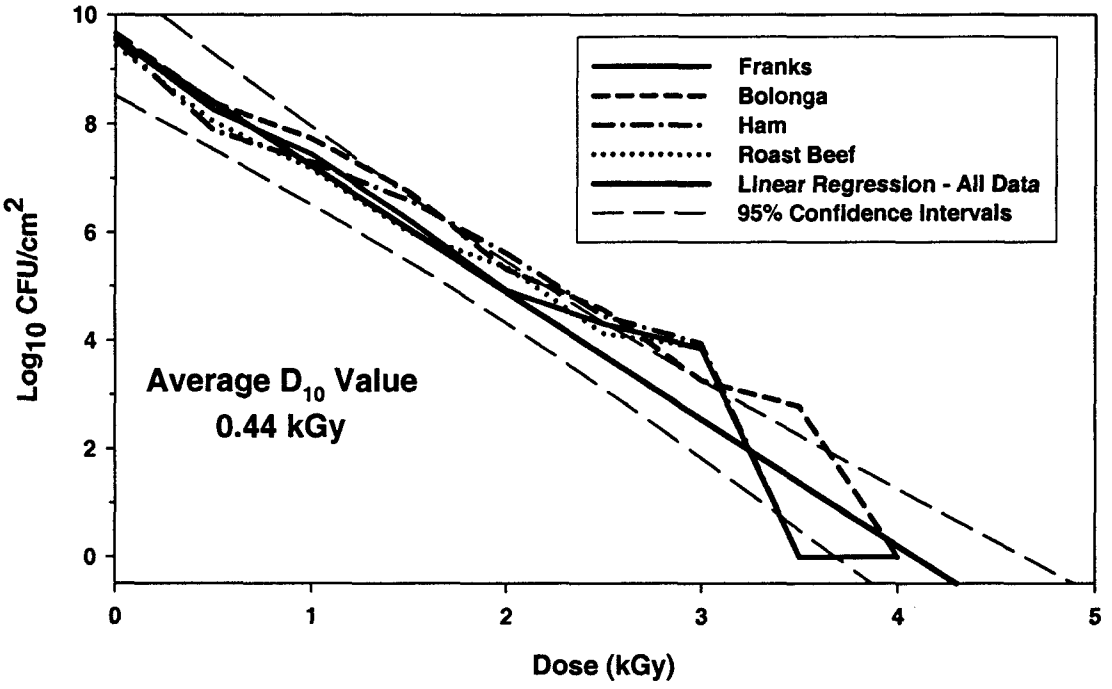


FIGURE 4.1. Survival of *Listeria monocytogenes* on irradiated RTE meats

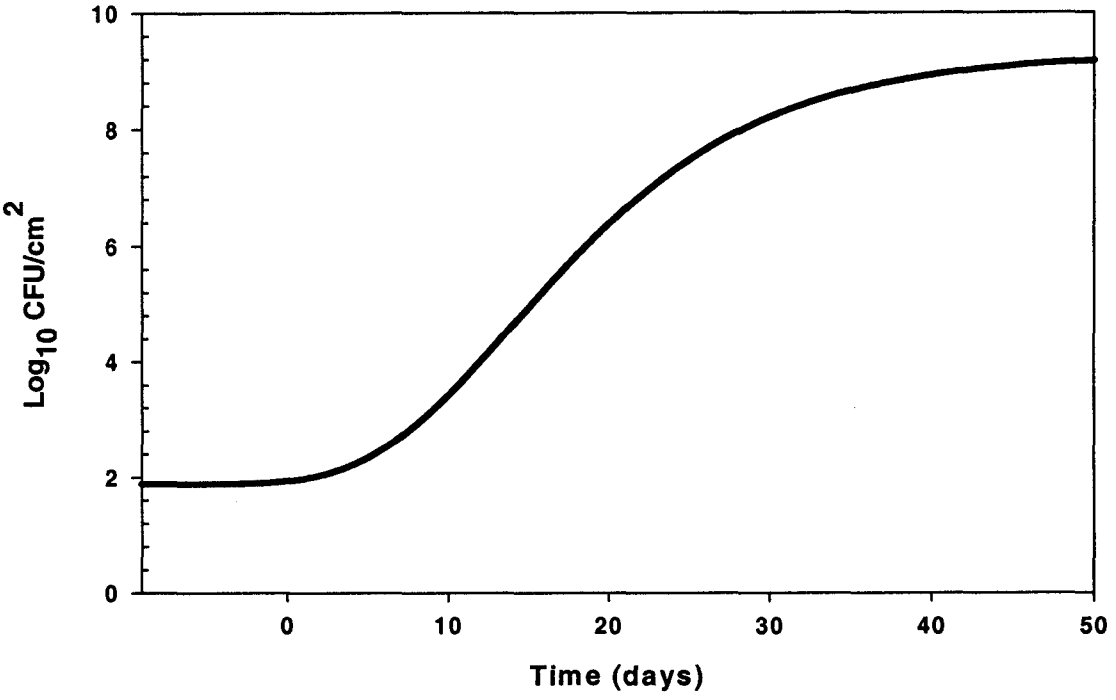
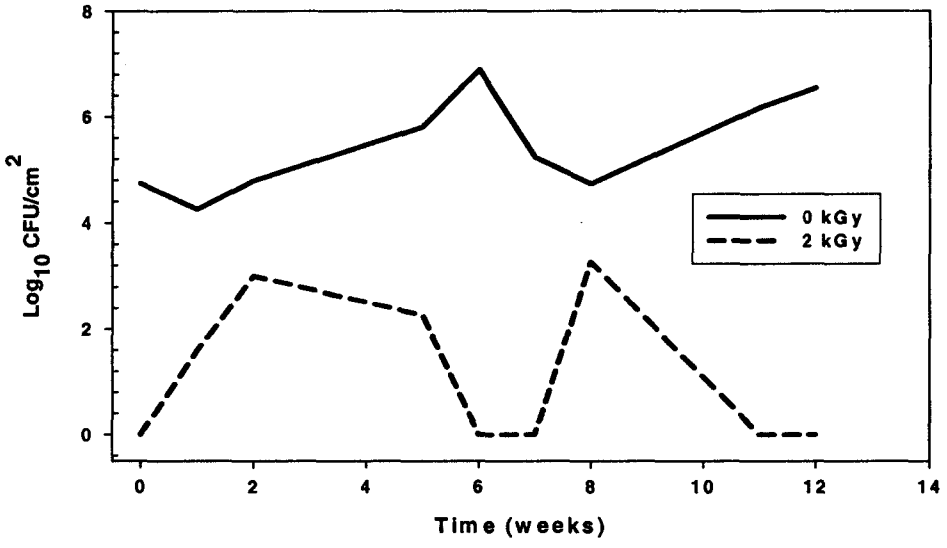


FIGURE 4.2. Projected growth of *L. monocytogenes* on RTE processed meats at 10°C [Pathogen Modeling Program (PMP)]

(a) 4°C



(b) 10°C

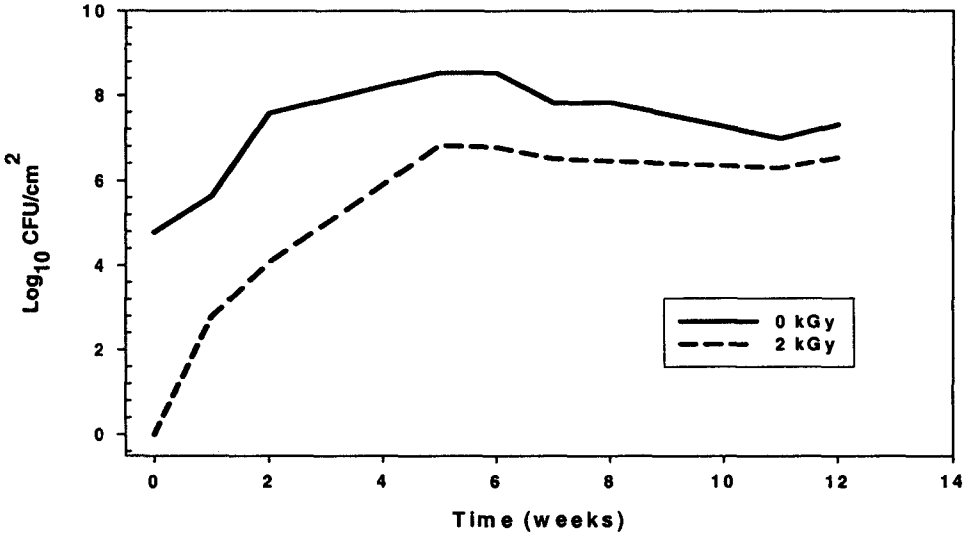
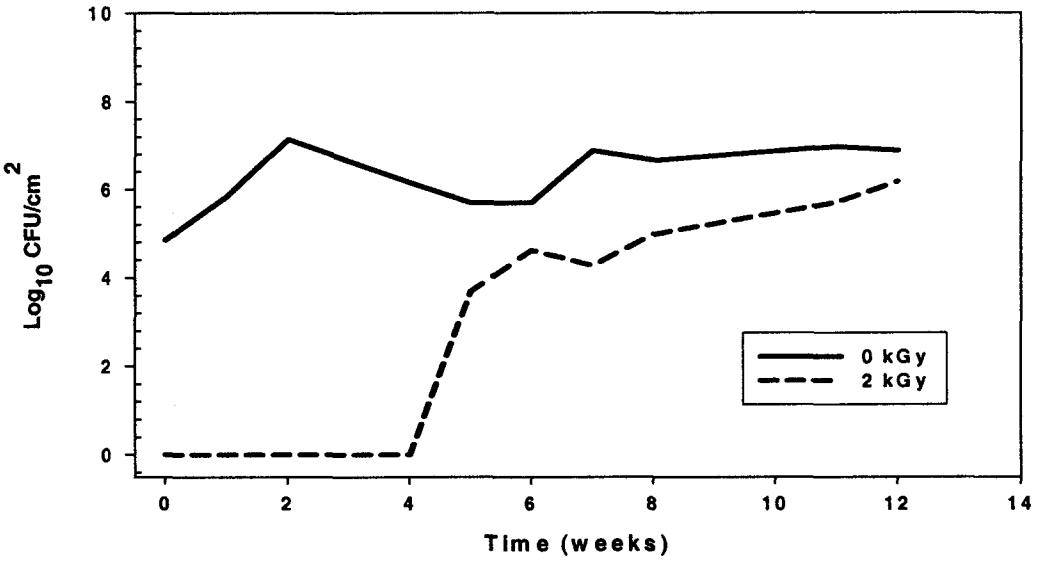


FIGURE 4.3. Survival and growth of *L. monocytogenes* on frankfurters based on absolute counts

(a) 4°C



(b) 10°C

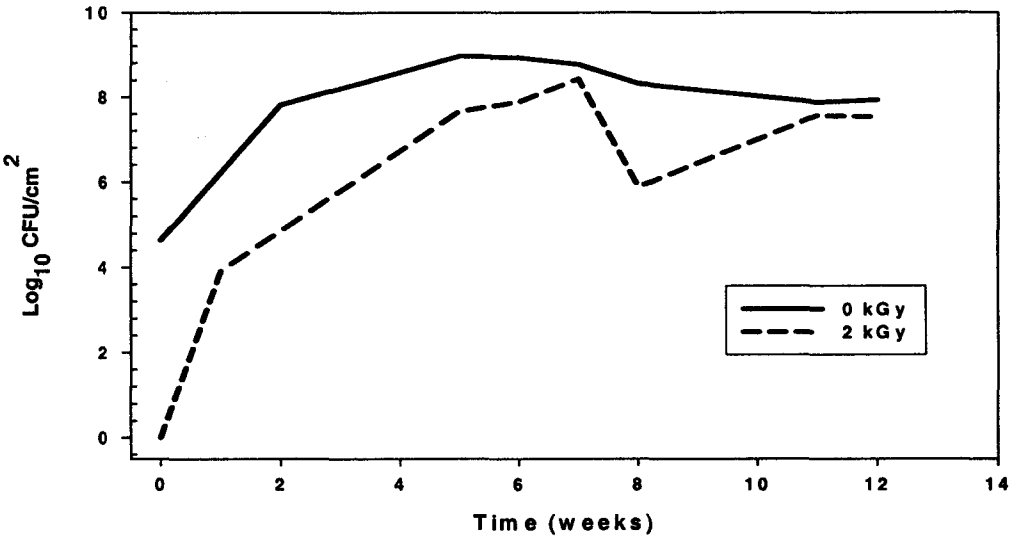
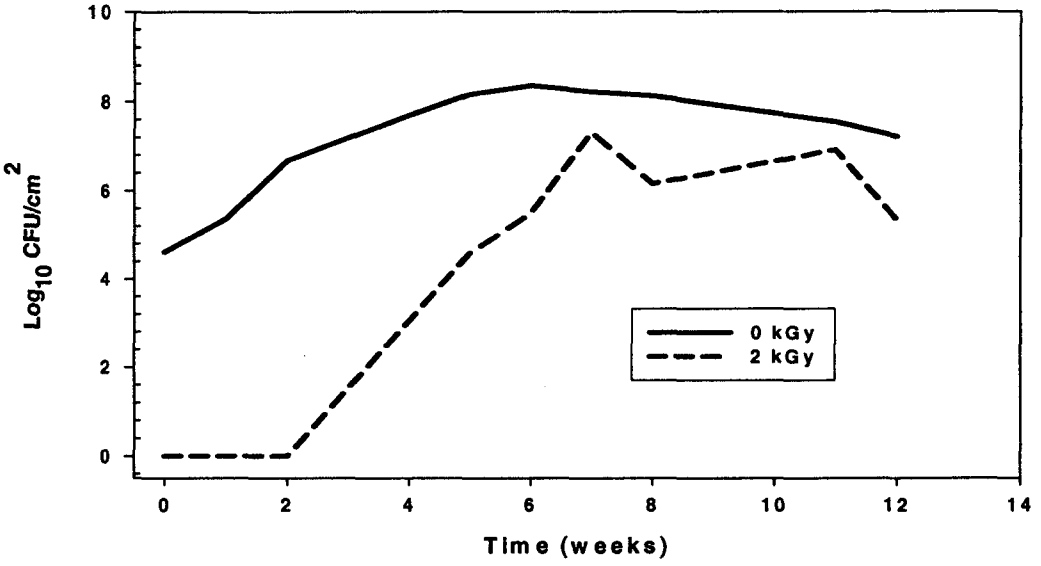


FIGURE 4.4. Survival and growth of *L. monocytogenes* on bologna based on absolute counts

(a) 4°C



(b) 10°C

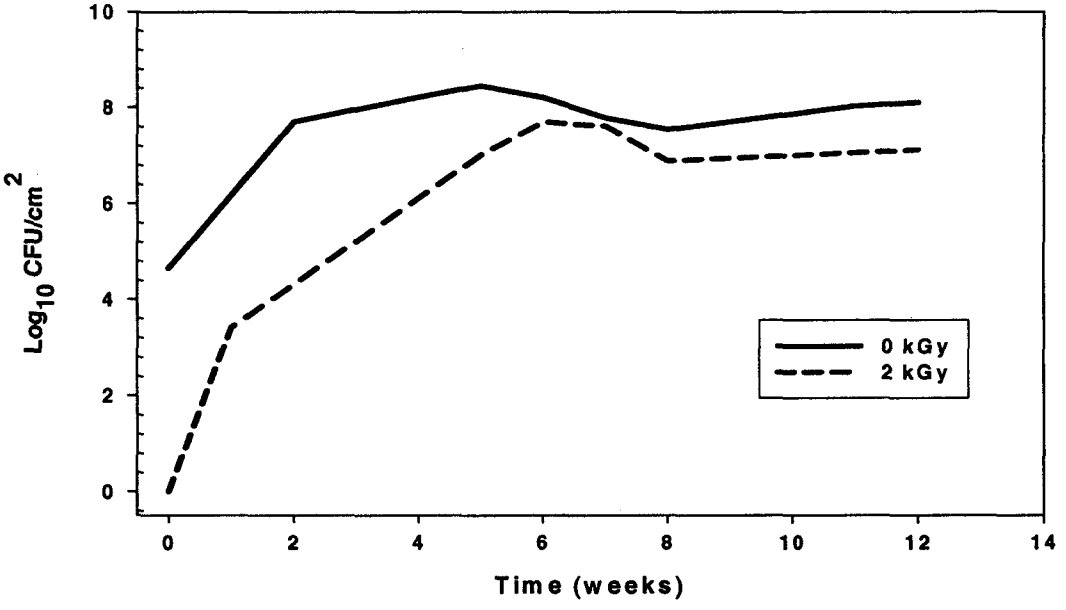


FIGURE 4.5. Survival and growth of *L. monocytogenes* on turkey ham based on absolute counts

CHAPTER 5.

SENSORY EVALUATION OF IRRADIATED AND NONIRRADIATED READY-TO-EAT (RTE) MEATS

A paper to be submitted for publication in the Journal of Food Science

S.C.C. FOONG, J.A. LOVE, AND J.S. DICKSON

ABSTRACT

Four types of selected ready-to-eat (RTE) meats were irradiated at a targeted dose of 1.5 kGy, which provided a 3-log reduction of inoculated *Listeria monocytogenes* (10^{10} CFU/ml). The meats selected were commercially available frankfurter, sliced chopped ham, sliced light bologna, and sliced oven roasted cured beef. A low dose irradiation provided a margin of safety and at the same time minimized organoleptic changes. Human subjects (120) participated in a triangle test sensory evaluation on whether the odd sample was chosen. Frankfurters showed no significant difference ($P > 0.05$) while the sliced meats showed significant differences ($P < 0.05$). Based on the judgments, consumers had a more difficult time choosing between irradiated and nonirradiated frankfurters but relatively easy for the irradiated and nonirradiated sliced meats.

Introduction

Irradiation is considered to be a relatively new technology in food safety and has been approved by the United States Department of Agriculture (USDA) for beef, pork, and poultry but not currently for processed meats (Bjerklie 1999). Irradiation is a means of controlling contaminants, namely pathogens, thus reducing public health hazards associated with ready-to-eat (RTE) meat products (Mitchell 1994; Bjerklie 1999). Besides microbial safety, irradiation also has the potential to extend storage life by decreasing the chances of post-processing contamination (Mitchell 1994; IFST 1999). Irradiation of processed meats, however, does change flavor, aroma, and color (Shay and others 1988). Consumer acceptance of irradiated products is increasing with increased efforts in reducing the misconception that irradiation makes food radioactive (IFST 1999). The overall goal for this study was to determine if consumers could tell a difference if RTE meats were irradiated. Experiments were conducted to test the hypothesis that there was no difference between the irradiated and nonirradiated frankfurter, ham, bologna, and oven roasted cured beef. The primary objective was to determine whether there was a difference between irradiated and nonirradiated selected meats using the triangle test.

Materials and Methods

Experimental design and sensory methods

Prior to any testing, permission was obtained from the Human Subjects Review Board of Iowa State University to conduct this experiment. A randomized order of sample presentation was used. Sample codes and orders of presentation to subjects were generated using computer software. A triangle test for consumers being able to detect a difference

between irradiated and nonirradiated frankfurters, ham, bologna, and roast beef was conducted. An analytical difference test was conducted to determine the proportion of consumers who can detect a difference between irradiated and nonirradiated selected meats. The variables and levels tested were the four different kinds of meat, control (nonirradiated) and irradiated at a target dose of 1.5 kGy (kiloGray). This dose was chosen based on a previous study that 1.5 kGy was required for a 3-$\log_{10}$ reduction of *Listeria monocytogenes* on selected RTE meats. Actual doses for frankfurters were 1.83 ± 0.11 kGy, ham 1.73 ± 0.07 kGy, bologna 1.70 ± 0.03 kGy, and roast beef 1.85 ± 0.01 kGy. The proportion values obtained (correct judgments) were statistically analyzed to determine at an alpha level of 0.05 whether to accept or reject the null hypothesis. Replications by subject were not conducted.

Panel

The number of replications was conducting a consumer test of n = 120. Since this was a test to determine difference, there were no specific criteria for selection of subjects for the consumer panel and no training was required. The study was conducted with volunteers on a first-come-first-serve basis. Subjects consisted of students, faculty, and staff at Iowa State University. The entire experiment was divided into 3 separate days to accommodate the large amount of samples and preparation time involved. Therefore, for each day, 40 people participated. Subjects who participated ranged from ages 18 to 72. Female subjects volunteered more often than males (62.5% vs 37.5%). For every subject, an informed consent statement was signed prior to taking the test. Various types of chocolates were available as a reward for participation.

Test conditions

Test area. The central location for this experiment was the lounge foyer area at LeBaron Hall, Iowa State University. Tables and chairs were set up for the consumer panel. The environment was not of an enclosed area and the test was conducted at building temperature with ample light source from fluorescent lights located above the ceiling in the hallway.

Sample preparation. One-ounce plastic sample cups were labeled on the side with three digit codes using a black wax pencil. The codes were generated randomly using computer software. The order in which the samples were presented was also randomized. Frankfurters were served at room temperature. Water was brought to a boil, the heat turned off, and the irradiated and nonirradiated frankfurters were immersed in different pots for 7 minutes. These frankfurters were removed and placed on separate cutting boards. A plastic measurement ruler was used to ascertain the length was 2.54 cm after the rounded ends were removed. The pieces of frankfurters were allocated into the 1 oz coded plastic cups. Plastic lid were put on immediately. These were then set out so that the temperature will equilibrate with the environment.

Sliced ham, bologna, and roast beef were served without any prior heat treatment. The sample size for ham and bologna were 3 pieces (2.54 x 2.54 cm^2 each) and roast beef was 4 pieces (2.54 x 2.54 cm^2 each). Slicing was carried out using separate cutting boards for irradiated and nonirradiated samples. The pieces of meat were allocated into their respective coded plastic cups and the lids were put on immediately. These cups were stored in the refrigerator until presentation.

Each subject performed a triangle test for irradiated and nonirradiated frankfurters, ham, bologna, and roast beef. Therefore, each subject had 12 samples to evaluate; 3 samples

of each of the 4 kinds of meat. Each subject was requested to sign the consent form prior to testing. Instructions for testing the triangle tests were stated on the score sheets. No carrier was used and time interval between samples was not specified. The subject was to taste the samples from left to right and from top to bottom. Subjects had the option of expectorating or consuming the samples but have to rinse their mouth or drink some water in between samples. The 3-digit code of the sample that they thought was the odd sample was written on the score sheet for each of the meats. Consent forms and score sheets were collected once the subjects were done. Copies of the consent form describing the study and contact information were available for the subjects in case they had further information.

Statistical Analysis

Analysis was carried out according to the equation for triangle test presented in Lawless and Heymann (1999). The minimum numbers of correct judgments for 120 total observations to establish significance at a probability level of 5% for the triangle test (one-tailed, $p = ⅓$) was determined using the formula:

$$X = 0.4714\ z(n)^{1/2} + [(2n+3)/6] \quad \text{where}$$

X = minimum number of correct judgments, n = number of trials (120), and $z = 1.64$ at a probability (α) equal to 5%. Therefore, the minimum numbers of correct judgments to establish significance at α value of 5% is 49 for a total of 120 observations.

Results and Discussion

Frankfurters

The correct judgments from the sensory evaluation conducted for frankfurters were 38 (Table 5.1). These correct judgments indicated the number of people being able to detect a difference in choosing the odd sample. The minimum of correct judgments required to establish significance at α value equal to 5% were 49. This value was much larger than the 38 correct judgments from the study, and therefore the panelists could not detect a difference between irradiated and nonirradiated samples. Frankfurters were heated prior to serving. The heat treatment decreased the differences between irradiated and nonirradiated frankfurters.

Sliced meats

Similar to frankfurters, the correct judgments from the sensory evaluation conducted for ham, bologna, and roast beef are shown (Table 5.1). The number of correct judgments, that is, the number of people being able to detect a difference in choosing the odd sample, for ham was 63, bologna was 65, and roast beef was 54. The minimum of correct judgments required to establish significance at α value equal to 5% were 49. This value was smaller than the correct judgments from the ham, bologna, and roast beef from the study. Therefore, the panelists could detect a difference between irradiated and nonirradiated sliced meat samples. These sliced meats are often consumed as cold cuts in sandwiches, thus, no prior heat treatment was carried out before presenting the samples to the panelists. Differences were detected when the sliced meats were consumed by without condiments. These

differences might not be detected if the cold cut meats were tested in sandwich form with bread and cheese with other trimmings and condiments.

Conclusions

The purpose of this study was to determine if consumers were able to detect a difference between irradiated and nonirradiated frankfurters, ham, bologna, and roast beef. No significant difference ($P > 0.05$) was observed for frankfurters but differences ($P < 0.05$) were observed for ham, bologna, and roast beef. These meats were presented to the panel based on the normal preparation of these RTE meats prior to serving. As frankfurters were usually boiled or grilled and consumed with bread and other condiments, the irradiated sample might not be differentiated. However, sliced meats are usually consumed in cold cut sandwiches. Without prior heat treatment, a distinction between irradiated and nonirradiated samples might be detected. Perhaps upon consumption as is in sandwiches with bread, cheese, and other trimmings might mask the very slight differences in taste.

When conducting this experiment, care must be taken in preparing these perishable RTE meats. Constant temperature monitoring is highly recommended to avoid temperature abuse of the products making them unsafe for consumer testing. On the same note, products should be well within the expiration dates to allot for the time frame required to conduct irradiation run and the entire consumer testing. For future testing, perhaps the test could be conducted with bread or cheese simulating sandwiches, which would probably decrease the chances of detecting a difference between irradiated and nonirradiated RTE meats.

Acknowledgments

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Table 5.1 - Number of subjects making correct judgments in detecting a difference between irradiated and nonirradiated RTE meats

RTE Meats	Correct	Incorrect
Frankfurters	38	82
Ham	63	57
Bologna	65	55
Roast Beef	54	66
Total	220	260

CHAPTER 6.

GENERAL CONCLUSIONS

Listeria monocytogenes is ubiquitous in the environment. Often times, this organism was found on or in animals, plants, soil, food contact surface, drains, and pipes. Survival in dust and condensate were questionable. This research indicated that *L. monocytogenes* could survive in simulated dust (sand) for more than 70 days and condensate for fewer than 21 days (*3*). Higher recovery of *L. monocytogenes* was obtained at lower temperature (10°C) and relatively higher humidity (88% RH). The study also showed that if dust contaminated with *L. monocytogenes* ended up on processed ready-to-eat (RTE) meats, this pathogen was able to survive and grow within 24 h. Even though prior assumption was made that the different processing types of RTE meats might affect the differences in survival of *L. monocytogenes*, no significant differences ($P > 0.05$) were found among them. The RTE meats chosen were frankfurters (intact comminuted meat), bologna (similar to frankfurters but sliced), ham (cured), and roast beef (intact muscle). Preliminary studies of scanning electron micrographs showed that *L. monocytogenes* existed in sand without nutrients after four months of storage at four different conditions. This organism survived in single cells with no observed clusters (Fig. 6.1).

Upon completion of this environmental survival of *L. monocytogenes*, whether this pathogen goes into the viable but nonculturable (VBNC) state affecting plate counts was questioned. Another concern was if appropriate media were used to generate reliable data due to the stressed conditions involved. Sand inoculated with *L. monocytogenes* was stored at 4°C and samples taken every two weeks for analysis for a period of two months. The initial four weeks showed formation of this state but only one VBNC cell was observed in a

microscopic field along with hundreds of other viable culturable cells. Frequency of finding VBNC cells in a microscopic field was most often during the second and fourth week. The last four weeks showed no signs of VBNC cells. Microscopic fields illustrated viable culturable cells and dead cells only. These results indicated that *L. monocytogenes* did not form VBNC cells well, thus, standard plate counts were adequate in obtaining reliable data on recovering stressed cells. In regards to the best nonselective medium of choice to recover desiccated stressed *L. monocytogenes* cells, tryptic soy agar with 0.6% yeast extract (TSAYE) was still better. This medium provided reliable results as well as being economical. Addition of pyruvate to the medium helped but did not provide a significant difference. Anaerobic incubation did not demonstrate better recovery of stressed listerial cells as previously reported (*5, 10*).

Understanding the attachment mechanism of *L. monocytogenes* on RTE meats after learning that this pathogen could survive in the environment is important. Among the five individual strains and a mixed cocktail of all five studied, no significant differences ($P > 0.05$) in attachment was observed. Preliminary study on their respective growth curves indicated similar lag phase duration and generation times (Table 6.1).

L. monocytogenes (ca. 86%) was able to attach strongly to RTE meats within 5 min. Increased attachment was observed with an increase in cell surface hydrophobicity (2). Based on the results from the hydrophobic ion chromatography (HIC), cationized ferritin staining, and electrostatic ion chromatography (ESIC), the different strains showed similar cell surface hydrophobicity and cell surface charge characteristics. Therefore, the strains used in this experiment would attach similarly to surfaces. However, using the 5 strain mixed culture to study effects of culture age in attachment, scanning electron micrographs

showed variation in the distribution of cell surface negative charge which was decreased on day 3 but increased on day 7. *L. monocytogenes* on RTE meats changed surface hydrophobicity of RTE meats. This change depended on the types of RTE meat. Whole intact frankfurters, upon heat treatment, has a surface skin that is more hydrophobic compared to the other sliced meats. *L. monocytogenes* is hydrophilic (*6*) and when inoculated onto the RTE meats, these surfaces became more hydrophilic compared to the uninoculated meats.

Many options are available in controlling this pathogen from processing plants that manufacture RTE meats. Avoidance of *L. monocytogenes* contamination from processed or cooked meats can be obtained when processors adhere to procedures for effective cooking, effective segregation of raw materials from cooked products, high standards of personal hygiene by training of operatives, and frequent effective cleaning and disinfection of product contact and other production areas post cooking (minimizing aerosol formation at all times). Presence of factors inhibiting growth in the product, well-controlled chill storage temperature, and reduced shelf-life also preclude opportunities for extensive growth (*1, 8*). Implementation of the Hazard Analysis and Critical Control Point (HACCP) is very important in reducing the occurrence of listerial contamination (*4*).

Irradiation is a means of preserving perishable foods like processed RTE meats. However, this application is not approved for RTE meats but highly recommended as these meats are consumed without any prior heat treatment. Most research conducted involved moderate to high dose irradiation which altered the organoleptic qualities negatively. With the selected RTE meats including sliced turkey, the D_{10} value for *L. monocytogenes* was 0.44 kGy. For a 3-$\log_{10}$ reduction, 1.5 kGy was required and for a 5-$\log_{10}$ reduction, 2.5 kGy.

Even though sodium lactate is widely used for sliced turkey for anti-listerial purposes, the same doses were required for the 3- and 5- $\log_{10}$ reductions.

Using low dose irradiation provides a margin of safety while maintaining aesthetic appeal and quality of RTE meats. On the downside, survival and outgrowth of *L. monocytogenes* is possible. An extended lag phase was observed with selected processed meats irradiated at 2 kGy and especially for the ones stored at 4°C. The lag phase for bologna was extended to 4 weeks and turkey ham for 2 weeks when irradiated at 2 kGy and stored at 4°C. This extended shelf life is important as irradiated meats can be stored for much longer time before spoilage. No recoverable bacterium was found at 4 kGy stored at either temperature (4°C or 10°C). Also, no apparent difference in generation time and maximum population densities was observed. Even though irradiation enhances safety of foods, the process cannot replace the effectiveness of good manufacturing practices and good production hygiene (*7*).

Once the low irradiation dose was established for a 3-$\log_{10}$ reduction of *L. monocytogenes*, the question remained if consumers could detect differences in processed RTE meats treated with irradiation to those without. The sensory evaluation conducted with the four selected meats using triangle tests provided data if consumers make correct judgments in choosing the odd sample. From the data gathered, consumers could not detect any differences ($P > 0.05$) between irradiated and nonirradiated frankfurters but could detect differences ($P < 0.05$) for the sliced meats. Boiling the frankfurters or some sort of heat treatment prior to consumption suppressed the slight differences. The sliced meats, bologna, ham, and roast beef, were much easier for consumers to detect the odd sample. Although, statistical significant differences were obtained, anecdotal comments from the panel

indicated that most of the time, they were just guessing, stating the 'test' was very difficult, and was questioning if all the samples were indeed the same but were set up to trick them.

Recommendations for Future Research

In the environmental study of *Listeria monocytogenes* surviving in dust and condensate, questions were raised in the matter of what the control was. Perhaps a smaller scale experiment can be conducted to compare stressed cells to healthy ones. A lot more work can be carried out for the VBNC study. For instance, actual counts from viable cells and correlate them with plate counts and work on different concentrations of antibiotics and observe the differences in elongation. More media types or their modifications can be used in comparing and obtaining reliable standard plate counts to recover stressed cells. A comparison study with different types of stresses and media most suitable in recovering injured cells can be conducted. Perhaps, addition of glutathione or Oxyrase® in minimal media may have better recovery (*9*).

For the attachment studies, food contact surfaces, such as, stainless steel, glass, plastic, and rubber, can be looked into as they have different surface properties that may enhance *L. monocytogenes* attachment, and forming biofilms that may potentially contaminate foods post-processing. Other kinds of processed meats of interest can be part of the study as well. Since many established tests are available for cell surface hydrophobicity, perhaps these can be standardized or correlated to obtain the best method for specific applications. A variety of applications can be carried out using the cationized ferritin stain to determine cell surface charge distribution. Definitely much more study is needed to understand the changes in cell surface charge with age, to answer why the charges decreased

at day 3 and increased again at day 7. Scanning electron micrographs also illustrated some sort of extracellular fibrils that might affect attachment. This issue should be looked into. A lot of variations were observed when the contact angle measurement (CAM) was conducted. Perhaps a better procedure can be developed to decrease these variations. The CAM results indicated that processed meat surfaces were indeed hydrophobic which would increase bacterial attachment. These surfaces can be manipulated to decrease hydrophobicity which in turn will decrease attachment.

D_{10} values obtained with irradiation are dependent on the bacterial species and meat types. Perhaps a difference in D_{10} values can be observed between rough and smooth strains of *L. monocytogenes*. Since processing formulations and procedures are so diverse in the world of processed meats, more variety of RTE meats can be used. The same goes for the study on survival and outgrowth of other pathogenic bacteria on RTE meats treated with low dose irradiation. Shelf-life will be extended but for how long will be dependent on the bacterial species, processed meat type, irradiation dose, and storage temperature. Other possible future directions can be investigating individual types of RTE meats, its respective formulation, and the effects of each ingredient on the antimicrobial activities against *L. monocytogenes*. Differences in formulations and processing procedures do affect the efficiency of other preventative methods to provide safer foods.

The sensory evaluation conducted provided important data but a lot more information can be gleaned from more detailed studies. For example, preference tests can be conducted. Informing the panels to tell a difference by taste alone may help instead of just a 'different' sample. This 'difference' could be misleading as the panels judged based on color, odor, or whichever sample that they preferred. Another form of sensory evaluation could be based on

the hedonic scale of like and dislike. Also, stating sensory attributes with an irradiated sample as a reference can also be another option in providing good information. Future research is to incorporate the meats with some bread or cheese to simulate exactly how these meats are consumed. Wine will be optional.

This study has provided immense information on how *L. monocytogenes* was able to survive in the environment to contaminating processed meats. Intervention by means of low dose irradiation is acceptable in providing added safety.

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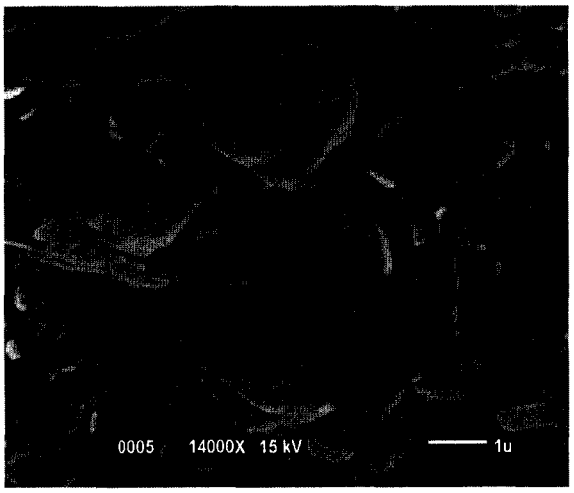
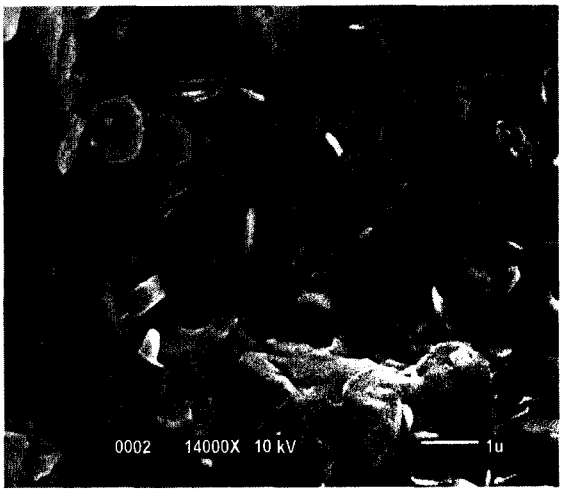
Table 6.1. Lag phase duration and generation times for *Listeria monocytogenes*

Strain	Lag phase duration (h)	Generation time (h)
Scott A	3.55	0.631
1/2a H7764	3.59	0.625
4b H7969	3.32	0.588
4b H7962	3.59	0.591
4b OB90393	3.58	0.595
Mixed culture	3.49	0.597

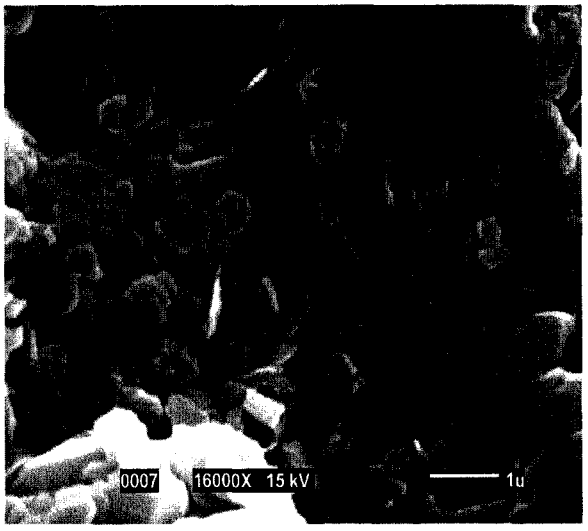
(a) 10°C at 88% RH



(b) 10°C at 0% RH



(c) 22°C at 40% RH



(d) 22°C at 0% RH

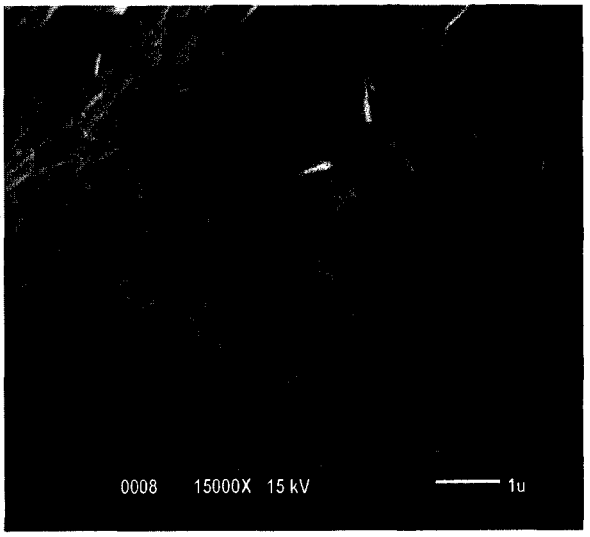


FIGURE 6.1. *Listeria monocytogenes* in sand without nutrients at four different conditions after four months of storage

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I DID IT!!!!! YEAH!!!!!

BIOGRAPHICAL SKETCH

Chi-Ching Foong (Sally) was born October 18, 1973 in Ipoh, Perak Darul Ridzuan, Malaysia. She received the Bachelor of Science in Food Science and Technology (Science Option) and Business Administration from University of Wisconsin at River Falls in 1995 and the Master of Science in Poultry Science from University of Arkansas at Fayetteville in 1999. She was listed in *Who's Who Among Students in American Universities and Colleges* in 1995. She has served as a Research Assistant in the Poultry Science Department at University of Arkansas and a Teaching/Research Assistant in the Department of Microbiology at Iowa State University.